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Mechanisms by which natural polyphenols regulate the expression of cytoprotective genes

Xiao, Han

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Han Xiao

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University of Dundee

Mechanisms by which natural polyphenols regulate the expression of cytoprotective genes

Han Xiao

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Abbreviations

ADH	Alcohol Dehydrogenase
NAT	N-acetyltransferase
3-MC	3-methylcholanthrene
AhR	Aryl hydrocarbon receptore
AhRR	AhR repressor
AKR	Aldo-keto reductase
AKR	Aldo-keto reductase (AKR)
AR	Androgen receptor
ARE	Antioxidant responsive element
bHLH	Basic helix-loop-helix
BTB	Bric-à-Brac
bZIP	Basic region-leucine zipper
CAR	Constitutive androstane receptor
CBP	CREB-bindg protein
CDK	Cyclin-dependent kinase
CHD	Chromodomain helicase DNA-binding
CHX	Cycloheximide
CNC	Cap'n'collar
COMT	Catechol-O-methyltransferase
Cul3	Cullin3
CYP	Cytochrome P450

DAPI	4'-6-diamidino-2-phenylindole
dd H_2O	Double distilled water
DDB1	Damaged-DNA-bind 1
DMEM	Dulbeccos's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Epo	Erythoropoietin
ER	Estrogen receptor
ERK	Extracellular signal-related kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	Glutamate cysteine ligase catalytic subunit
GCLM	Glutamate cysteine ligase modulatory subunit
GSK3β	Glycogen synthase kinase-3β
GST	Glutathione transferase
Hif1	Hypoxia-inducible factor 1
HSP	Heat-shock protein
I3C	Indole-3-carbinol
ICZ	indolo[3,2-b]carbazole

Ig	Immunoglobulin
IHC	Immunohistochemistry
IVR	Intervening region
ITE	2-(1'H-indole-3'-carbonyl) thiazaole-4-carboxylic acid methyl ester
ITS	Insulin-transferin-selenium
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1 (Keap1)
LB	Luria Bertani
Maf	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
MAPK	Mitogen-activated protein kinase (MAPK)
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF-E2 p45	nuclear factor-erthyroid 2 p45-subunit
NQO1	NAD(P)H dehydrogenase, quinone 1
Nrf	Nuclear factor erythroid 2 p45 subunit-related factor
OATP	Organic anion transporting polypeptide
P/S/T	Proline/Serine/Thereonine
P-gp	P-glycoprotein
PAH	Polycyclic aromatic hydrocarbon

PAS	Per-ARNT-Sim
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferation receptor (PPAR)
PXR	Pregnane X receptor
Rb	Retinoid binding
Rbx1	Ring-box 1
SAPK	Stress-activated protein kinase
SDR	Short Chain dehyrogenase/reductase (SDR)
SULT	Sulfotransferase
TAD	Transactivation domain
TBL3	Trasducin β-like 3
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TXN	Thioredoxin
UDP-GT	Uridine diposphate-glucuronyl transferase
UV	Ultraviolet
v/v	Volume / volume
XRE	Xenobiotic responsive element
β-gal	β-D-galactopyranoside

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Declaration

I declare that this thesis is based on results obtained from investigations which I have personally carried out, and that the entire thesis is my own composition. Any work other than my own is clearly stated in the text and acknowledged with reference to any relevant investigators or contributors. The thesis has never been presented previously, in whole or part, for the award of any higher degree. I have consulted all the references cited within the text of this thesis.

Signed …… Date …….....

I confirm that Han Xiao has spent the equivalent of at least 9 terms in the Biomedical Research Centre, University of Dundee, Ninewells Hospital and Medical School, and that she has fulfilled the conditions of the University of Dundee, thereby qualifying her to submit this thesis in application for the degree of Doctor of Philosophy.

Signed …… Date …….

Abstract

Epidemiological studies have shown that a diet high in fruit and vegetables has an inverse association with the occurrence of various degenerative diseases such as cancer, cardiovascular disease, neuro-degenerative disease and diabetes. Clinical and animal studies using fruit and vegetable extracts have shown that polyphenols abundant in plants may account for the beneficial effect of diets high in fruit and vegetables. Following these findings, laboratories worldwide are investigating the mechanisms underlying the health effects exerted by polyphenols. Although many studies have investigated the effect of various polyphenols on cancer cells, such as inhibition of cell growth, induction of apoptosis, tumorigenesis, few studies have been carried out to examine their effect on normal cells and whether they could prevent the initiation of cancer. DNA damage, one of the early steps in the tumorigenic process, may be caused by toxicants and oxidative stress either from the environment or from endogenous sources. Therefore, to prevent such damage, multiple mechanisms are deployed by cells to combat the toxic insult and reduce oxidative stress. The CNC-bZIP transcription factor, Nrf2, is known to regulate the expression of many of the genes involved in these processes.

Under homeostatic conditions, the Nrf2 protein is targeted by its inhibitor Keap1 for ubiquitination in the cytosol. Upon redox stress, induced by low molecular weight electrophiles, Nrf2 can evade ubiquitination and translocate into the nucleus, where it heterodimerizes with members of the small Maf family of transcription factors and

bind to the antioxidant response element (ARE) in the promoter region of many cellular defence genes, leading to the up-regulation of their transcription.

In our study, we examined whether polyphenols could affect the Nrf2-ARE signalling pathway. Preliminary screening was first carried out using AREc32 cells to find flavonoids which have ARE-inducing ability. Flavonoids, which are present in relatively high concentration in fruits and vegetables and consumed most commonly, were chosen for screening. Quercetin and kaempferol showed the highest ARE-inducing ability and these two compounds were subsequently were used to examine their effect on Nrf2 and its target gene Nqo1 in rat liver RL-34 and mouse embryonic fibroblast MEF cells. By Nqo1 enzyme activity assay, Western blotting, and Taqman experiments, our study showed that these two flavonoids increased the enzyme activity, protein expression and mRNA level of Nqo1. By using $Nrf2^{+/+}$ and $Nrf2^{-/-}$ MEFs, we found that such increases are Nrf2-dependent. The effect of quercetin and kaempferol on Nrf2 was therefore examined. Nrf2 protein, but not mRNA, was found to be elevated by quercetin and kaempferol. By cycloheximide-chase experiment, quercetin and kaempferol were shown to stabilize the Nrf2 protein by decreasing its turnover time. Furthermore, results from cellular fractionation and immunocytochemistry experiments showed that Nrf2 predominantly resides in nucleus under both normal and stressed conditions; the two flavonoids increased the accumulation of Nrf2 in both cytosol and nucleus, although the increase of Nrf2 protein in the nucleus was more pronounced. In addition, it was

found that the flavonoids inhibited the ubiquitination of Nrf2. To address how the flavonoids inhibit the ubiquitination, we carried out mutagenesis experiments with quercetin and found Cys151 in Keap1 is required for stabilization of Nrf2, which indicates that quercetin may act as an electrophile and modify the Cys151 in Keap1, ultimately leading to the disruption of the association between Keap1 and Nrf2. The involvement of ARE in the regulation of Nqo1 by quercetin and kaempferol were examined by mutagenesis experiments, the results of which showed that the ARE was involved in both the basal expression of Nqo1 and its induction by quercetin and kaempferol.

Besides the ARE, the promoter region of *Nqo1* contains a xenobiotic responsive element (XRE) which can mediate gene regulation by AhR. With the evidence from previous research showing that Nqo1 can be up-regulated by the AhR agonist TCDD, we examined the involvement of XRE in the induction of Nqo1 by the flavonoids in mutagenesis experiments. The absence of XRE only affected the basal level but not the inducible level of Nqo1. However, by immunocytochemistry experiments, we showed that quercetin and kaempferol can act as AhR agonists. This was confirmed by Taqman experiments showing these two flavonoids can increase the mRNA level of *Cyp1a1*.

Lastly, the effect of quercetin and kaempferol *in vivo* was also examined in animals by using C57BL/6 male mice. Only quercetin showed up-regulation of *Cyp1a1*

mRNA in the small intestine. An effect on Nqo1 and Nrf2 by the flavonoids observed in cells was not seen in the tissues.

Taken together, the data presented in this thesis shows that the flavonols quercetin and kaempferol up-regulate the Nrf2-ARE signalling pathway by stabilizing the CNC-bZIP protein; such up-regulation also leads to the trans-activation of *Nqo1*. In addition, the two flavonoids are AhR agonists and increase the mRNA level of *Cyp1a1* which is also observed in the mouse small intestine. Such increase of *Cyp1a1* may indicate their implication in the prevention of gastrointestinal cancer.

1 Introduction

1.1 Phytochemicals and their preventive effect

1.1.1 Phytochemicals and its subclasses

Phytochemicals are non-nutritive constituents produced by secondary metabolism in plants. They defend plants against predators, microbial infections and ultraviolet light, regulate metabolic pathways in plants and provide color, flavor and smell to the plants. Phytochemicals can be classified as carotenoids, polyphenols, alkaloid, nitrogen-containing compounds, and organosulfur compounds. Polyphenols are compounds possessing one or more aromatic rings with one or more hydroxyl group and are generally categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Liu, 2004).

1.1.2 Polypheonols

Polyphenols are abundant components in our diet and evidence for their role in the prevention of degenerative diseases such as cancer, cardiovascular and Parkinson's or Alzheimer's disease is emerging. In edible plants, several hundred molecules possessing polyphenol structure (i.e. several hydroxyl groups on aromatic rings) have been identified. In non-edible plants, even more of these related molecules have been identified, numbering several hundreds of them. Depending on the number of phenol rings and different types of connection between these rings, several groups of

polyphenols can be distinguished, including the phenolic acids, flavonoids, stilbenes, and lignans (Manach *et al.*, 2004).

1.1.3 Subclasses of flavonoids and their distribution in food

More than 4000 distinct flavonoids have been identified and approximately 900 of them are consumed in the human diet. As shown in Figure 1.1, flavonoids share a generic structure, consisting of two aromatic rings (A and B rings) linked by 3 carbons atoms that are usually contained in an oxygenated heterocycle ring, or C ring. The flavonoids can be further classified as flavonols, flavones, catechin-tanins, anthocyanidins, and isoflavones, according to structural differences in their C-ring (Table 1.1). Flavonoids are found in nearly all fruit and vegetables and are frequently found in nature as conjugates in gycosylated or esterified forms. A large number of different sugars, of which more than 80 kinds exist, also contribute to the chemical variety of flavonoids. However, flavonoids can occur as aglycones in nature and food processing can assist the conversion of conjugates to their aglycones. (Liu, 2004)

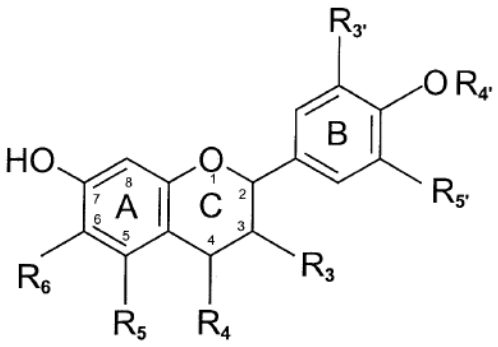


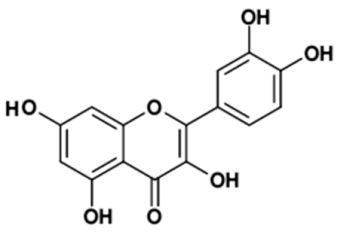
Figure 1.1 General structures of common food flavanoids.

Flavonoids class	Flavonols	Flavones	Isoflavones	Catechins	Anthocyanins
Carbon atom in ring C to which B is attacthed	2	2	3	2	2
C-ring unsaturation	2-3 double bond	2-3 double bond	2-3 double bond	None	1-2, 3-4 double bond
C-ring functional groups	3-hydroxy, 4-Oxo	4-Oxo	4-Oxo	3-hydroxy; 4-gallate	3-hydroxy

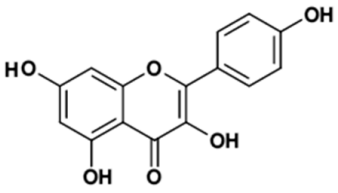
Table 1.1 Chemical characteristics of each flavonoid subclasses.

1.1.3.1 Flavonols

Flavonols are present in various foods, the structures of which are characterized by the presence of a double bond between C2 and C3 and a hydroxyl group in the C3 position (Figure 1.2). They are generally present at relatively low concentrations of around 15-30 mg/kg fresh weight and the richest sources are onions (up to 1.2 g/kg fresh wt), curly kale, leeks, broccoli, and blueberries. Amongst beverages, the amount of flavonols in red wine and tea can also be as great as 45 and 30 mg/L, respectively (D'Archivio *et al.*, 2007).



Quercetin

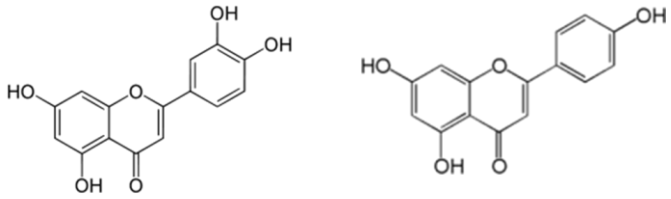


Kaempferol

Figure 1.2 Structures of the main representatives of flavonols quercetin and kaempferol

1.1.3.2 Flavones

Flavones are much less abundant than flavonols in fruit and vegetables and they characteristically possess a double bond between C2 and C3 as shown in Figure 1.3 and Table 1.1. Flavones consist chiefly of glycosides of luteolin and apigenin. Amongst edible plants, parsley and celery are important sources of flavones. However, polymethoxylated flavones can be found in large quantities in the skin of citrus fruits (D'Archivio *et al.*, 2007).



Luteolin Apigenin

Figure 1.3 Structures of typical flavones luteolin and apigenin.

1.1.3.3 Flavanones

High concentrations of flavanones are mainly found in citrus fruit, though they can also be found in tomatoes and aromatic plants such as mint. Their structures are characterized by the presence of a saturated three-carbon chain and an oxygen atom attached to the C4 atom (Figure 1.4 and Table 1.1). They are also present in the plant in their glycosylated form. The representative flavanones are naringenin, hesperetin and eriodictyol (D'Archivio *et al.*, 2007).

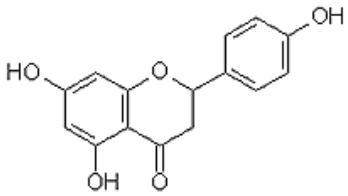


Figure 1.4 Structure of the typical flavanone narigenin.

1.1.3.4 Isoflavones

With the hydroxyl group in the C7 and C4 position, isoflavones share structural similarities to estrogen. Due to this property, the metabolites of isoflavones have been found to be able to bind to estrogen receptors and therefore have been classified as phytoestrogen (Reinli & Block, 1996). Soya and its processed products are the main source of isoflavones in the human diet. Like other flavonoids, they mostly exist in a glycosylated form. They are represented in plants as three main molecules: genistein, daidzein and glycitein (D'Archivio *et al.*, 2007).

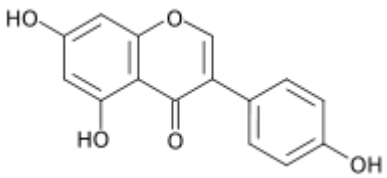


Figure 1.5 Structure of genistein, one of the typical isoflavones

1.1.3.5 Catechins

Catechins are found in many types of fruit such as apricot and cherry. However, green tea and chocolate are much greater sources of catechins. Unlike the flavonoids mentioned above, the catechins have a saturated three-carbon chain with a hydroxyl group in the C3 atom (Figure 1.6). Another distinguishing property of this family is

that they exist as aglycones in foods. Catechin and epicatechin are the main flavanols in fruit (D'Archivio *et al.*, 2007). Gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes and more importantly in tea (Manach *et al.*, 2004).

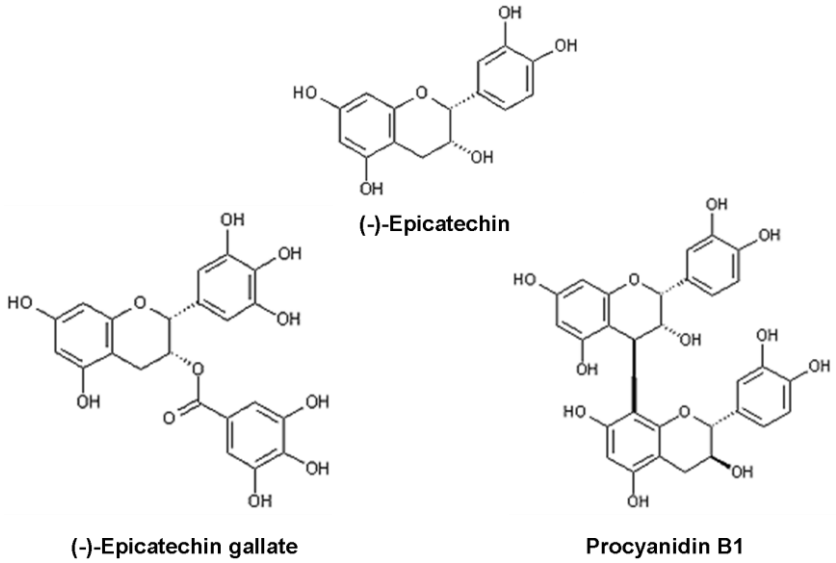


Figure 1.6 Structures of typical catechins.

1.1.3.6 Anthocyanins

Anthocyanins are water soluble and exist primarily in glycosytes which are called anthocyanidins. They serve as pigments that give rise to the red and blue colors in some fruits and vegetables. In human diet, anthocyanins are found in red wine, certain varieties of cereals, and certain leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), but they are most abundant in fruit. Cyanidin is the most common anthocyanidin in foods (Manach *et al.*, 2004).

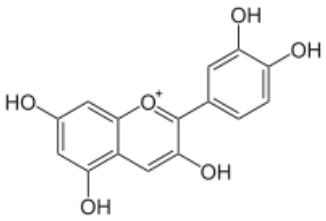


Figure 1.7 The structure of cyanidin.

Human intake of all flavonoids is estimated at a few hundred milligrams to a maximum of about 650 mg/day. The total average intake of flavonols (quercetin, myricetin , and kaempferol) and flavones (luteolin and apigenin) was estimated as 23 mg/day in the United States, Denmark and Hollond (Manach *et al.*, 2004). In Holland, quercetin contributed to ~70%; kaempferol, 17%; myricetin, 6%; luteolin, 4%; and apigenin 3% (Hertog *et al.*, 1993). However, this can vary a lot due to different areas.

Flavonoids		Source	Polyphenol content
			(mg/kg) fresh wt (or mg/L)
Anthocyanins	Cyanidin Pelargonidin Peonidin Delphinidin Malvidin	Aubergine	7500
		Blackberry	1000-4000
		Black currant	1300-4000
		Blueberry	250-5000
		Black grape	300-7500
		Cherry	350-4500
		Rhubarb	2000
		Strawberry	150-750
		Red wine	200-350
		Plum	20-250
		Red cabbage	250
Flavonols	Quercetin Kaempferol Myricetin	Yellow onion	350-1200
		Curly kale	300-600
		Leek	30-225
		Cherry tomato	15-200
		Broccoli	40-100
		Blue berry	30-160
		Black current	30-70
		Apricot	25-50
		Apple	20-40
		Beans, greent or white	10–50
		Black grape	15-40
		Tomato	2–15
		Black tea infusion	30-45
		Green tea infusion	20-35
		Red wine	2–30
Flavones	Apigenin Luteolin	Parsley	240-1850
		Celery	20-140
		Capsicum pepper	5–10
Flavanones	Hesperetin Naringenin Eriodictyol	Orange Juice	215-685
		Grapefruit juice	100-650
		Lemon juice	50-300
Catechin-tannins	Catechin Epicatechin	Chocolate	460-610
		Beans	350-550
		Apricot	100-250
		Cherry	50-220
		Grape	30-175
		Peach	50-140
		Blackberry	130
		Apple	20-120
		Green tea	100-800
		Black tea	60-500
		Red wine	80-300
		Cider	40

Table 1.2 Sources content of flavonoids in common foods.

The amount of representative flavonoids, from different family, present in different fruit and

vegetables are shown in table. Data are adapted from Manach *et al*. (2004)

1.1.4 Bioavailability of flavonoids

Bioavailability of flavonoids varies amongst different chemicals depending on their chemical structure, sugar groups attached and their molecular weight. For instance, the aglycone is more easily absorbed than their glycosides; proanthocyanidins existing as polymers and having high molecular weight are poorly absorbed in the small intestine and are rapidly metabolized and eliminated (Manach *et al.*, 2005). Direct evidence on the bioavailability of phenolic compounds has been obtained by measuring their concentration in plasma (Marrugat *et al.*, 2004) and urine (Tian *et al.*, 2006) after ingestion of either pure compounds or food stuffs in which the content of interested polyphenols have been determined (Fito *et al.*, 2007). Data have been reported showing that the maximal plasma concentrations of flavonoids are low, usually less than 1 μmol/L, reaching to a maximum level 1-2 h after ingestion. Therefore, the maintenance of a high concentration in plasma requires repeated ingestion of the polyphenols over time. (D'Archivio *et al.*, 2007). Studies investigating the extent of polyphenol absorption in humans, after the ingestion of a single dose of polyphenols provided as a pure compound, plant extract or whole food/beverage, showed that the quantities of intact polyphenols found in urine vary form one flavonoid to another. Inter-individual variations have also been observed, probably due to the different composition of the colonic microflora which can affect their metabolism (Manach *et al.*, 2005).

1.1.5 Absorption of polyphenols

To explore whether flavonoids can exert chemopreventive effects, it is more important to understand how they are absorbed by the body and whether they are further metabolized to biologically active or inactive metabolites. There are numerous sites important for the metabolism of dietary polyphenols, including the gastrointestinal tract, the liver, and various other tissues such as the skin and brain. In addition, flavonoids are able to penetrate tissues, particularly those in which they are metabolized such as liver and intestine. However, not many data are available in either humans or animals. In one study tea polyphenols were measured in human prostate tissue and showed that after consumption of 1.42 L of green tea or black tea for 5 days, catechins from the beverage were bioavailable in prostate tissue samples ranging from 21-107 pmol/g tissue (Henning *et al.*, 2006).

The route of absorption and metabolism of polyphenols is stomach, gastrointestinal tract and liver. After crossing these barriers, polyphenols will be circulated in the plasma and transported to various target tissues or excreted in either the urine or bile. Though the aglycones can be absorbed by small intestine, the most abundant forms of flavonoids in foods are esters, glycosides, or polymers which are poorly absorbed (Scalbert & Williamson, 2000). However, these conjugates of aglycones can be hydrolyzed by enzymes such as glycosidases which can occur in food itself (endogenous or during processing) or in the cells of the gastrointestinal mucosa or can be secreted by the colon microflora. Such reactions do not occur under the acidic

conditions in the stomach. The converted polyphenols are readily bioavailable to the body. After hydrolysis in the gastrointestinal tract, the aglycones are absorbed by the intestinal enterocyts where they undergo different conjugation reactions including glucuronidation by UDP-glucuronyl transferase (UDP-GT), methylation by catechol-*O*-methyltransferase (COMT). Once flavonoids reach the liver, any remaining aglycone will undergo glucuronidation or sulfation and methylated polyphenolics may undergo demethylation (Singh *et al.*, 2008). In addition, as flavonoids have a planar aromatic structure, they may undergo oxidation in their role as antioxidants to form quinone-like structures that are detoxified by conjugation with glutathione or are broken down to smaller phenolic compounds (Corona *et al.*, 2006).

Polyphenol metabolites circulate in the blood bound to proteins, and albumin represents the primary protein responsible for binding and transporting polyphenols. The affinity of polyphenols for albumin varies according to their chemical structure and it is not clear whether binding to albumin affects their biological activity (D'Archivio *et al.*, 2007).

1.1.6 Beneficial properties of flavonoids

Various potentially beneficial properties of dietary flavonoids have been proposed including antioxidant properties, chelation of metals, anti-viral activity, anti-bacterial activity, anti-inflammatory properties, oestrogenic activity, anti-mutagenic activity,

and either the activation or inhibition of various enzymes. Some flavonoids show strong antioxidant activity, such as quercetin and the anthocyanin aglycone, cyanidin, which was reported to have antioxidant potentials 4-fold higher than that of the vitamin E analogue, trolox (Rice-Evans *et al.*, 1995). There are basically three requirements for possessing antioxidant activity by flavonoids: firstly, the presence of ortho-dihydoxyl (catechol) groups on the B ring; secondly, the presence of a 2,3-double bond in the C ring; thirdly, free hydroxyl groups on the 5 and 7 position of the A ring (Apak *et al.*, 2007; Zhang, 2005). Their antioxidant activities have been suggested to be responsible for their cancer prevention property (Akira *et al.*, 2008; Padhye *et al.*, 2010). Some flavonoids, such as quercetin (Ferrali *et al.*, 1997), cyanidin and procyanidin (Quesada *et al.*, 2010) are good chelators of metals like iron, zinc and copper and therefore could inhibit platelet aggregation and therefore contribute to the prevention of cardiovascular disease (Mladěnka *et al.*, 2010). Furthermore, some flavonoids are able to affect the expression or activity of various enzymes. For example, quercetin and kaempferol have been reported to increase the activity of thioredoxin reductase in normal human keratinocytes (Sugahara *et al.*, 2010).

1.1.7 Flavonoids and disease

Epidemiological studies have shown that there is an inverse relationship between intake of flavonoids and the risk of developing cardiovascular disease, and age-related disease such as Alzheimer's disease, and various types of cancer (Singh

et al., 2008). However, the mechanisms responsible for their beneficial effect is still unclear and under intensive investigation. One of the suggested mechanisms is that flavonoids are protective through their antioxidant property. As sustained elevated levels of reactive oxygen species have been found to be associated with various neoplastic diseases, the antioxidant property and ability of flavonoids to induce cytoprotective enzymes may contribute to their chemopreventive effect.

Anti-inflammatory activity is another possible chemopreventive mechanism, as inflammation contributes to the initiation and progression of various neoplastic diseases (Pierini *et al.*, 2008). It has been reported that flavonoids extracted from fruit and vegetables can inhibit the NF-κB signalling pathway, which is involved in the initiation of inflammation (Prasad *et al.*, 2010). Furthermore, modulation of critical cell activities of cellular responses, by flavonoids, such as regulation of cell cycle (Ong *et al.*, 2010), cell proliferation and cell apoptosis (Zhong *et al.*, 2010), may also account for the chemopreventive effect of flavonoids.

1.1.8 Flavonoids and cancer

Although carcinogenesis is a complex multistep process, in which numerous molecular mechanisms play different roles (Hanahan & Weinberg, 2000) and six general steps are involved. These include initiation, promotion, progression, angiogenesis, invasion and metastasis. Flavonoids may reduce the cancer risk both by blocking initiation of carcinogenesis and by suppressing the later promotion and

/or suppression stages.

Regarding the initiation of carcinogenesis, it always starts with genetic alterations. To avoid this, direct and indirect strategies can be taken, i.e. preventing DNA attack by reactive oxygen species, reducing the toxicity of procarcinogens by enhancing their conjugation and excretion, inhibition of carcinogen uptake into cells, increased efflux of carcinogen from cells, and enhancing DNA repair (Manson *et al.*, 2007). For example, quercetin has been reported to protect DNA damage by hydrogen peroxide and benzo[a]pyrene (Duthie *et al.*, 1997; Wilms *et al.*, 2005).

The progression of cancer could also be halted by activation of cell cycle arrest or apoptosis. A number of flavonoids alone, or in combination, have been found to suppress cell proliferation or induce apoptosis in carcinoma cells including quercetin (Hsieh & Wu, 2009), EGCG, resveratrol (Hsieh & Wu, 2008), kaempferol (Leung *et al.*, 2007), procyanidin and pomegranate extracted ellagitannins (Syed *et al.*, 2007). EGCG has also been shown to inhibit cell growth by inducing cell cycle arrest in the G_0/G_1 phase (Chen *et al.*, 2004). Another flavonol found in rice bran, tricin, was shown to inhibit the growth of breast tumour cells by causing G_2/M arrest *in vitro* and *in vivo* (Cai *et al.*, 2004). Therefore, different flavonoids may exert their chemopreventive effect through distinct mechanisms.

1.2 Drug-metabolizing enzymes

1.2.1 The Drug-metabolizing enzyme system

Drug-metabolizing enzymes, also called xenobiotic transformation enzymes, provide the defence against foreign chemicals, to which humans are constantly exposed. Xenobiotics include a broad spectrum of chemicals, either manufactured or natural, such as drugs, pollutants, alkaloids and pyrolysis products found in cooked food. Many xenobiotics are toxic and if they accumulate in the body, they may cause cell damage and eventually kill an organism. Therefore, a large number of enzymes with various specificities are required for the biotransformation and elimination of these toxicants. Examples of reactions and enzymes involved in detoxification include: oxidation reaction catalyzed by cytochromes P450, alcohol dehydrogenase (ADH), aldehyde dehydrogenase and glutathione peroxidase; reduction reaction catalyzed by aldo-keto reductase (AKR), short chain dehydrogenase/reductase (SDR), and NAD(P)H dehydrogenase, quinone 1 (NQO1); hydrolysis catalyzed by epoxide hydrolase; conjugation reactions catalyzed by UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), methyltransferase, acetyltransferase, and glutathione transferase (GST) (Zimniak, 2008). Generally, the first three reactions mentioned above can introduce a functional group to the substrate such as –OH, -NH_2, -SH or –COOH leading to a modest increase in the hydrophilicity in the end-product. Conjugation reactions include glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione, and conjugation with amino acids. Cofactors are also required for these reactions; they react with functional groups originally present in

the substrate or introduced through the other types of detoxification reactions. Compared with oxidation and reduction, conjugation reactions can result in a significant increase in the hydrophilicity of the substrates, therefore promoting the excretion of foreign chemicals (Klaassen, 2008). In the 1970's the concept of Phase I and Phase II drug metabolism was proposed, in which the Phase I enzymes included those responsible for hydrolysis, oxidation, and reduction of xenobiotics while Phase II enzymes catalyze the conjugation of xenobiotics with sugars and amino acids (Williams, 1971).

In addition to phase I and II enzymes, transporters that can facilitate drug metabolizing have been classified as the phase III drug-metabolizing enzymes. The phase III transporters include a large family of proteins such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP) and organic anion transporting polypeptide. They are present in various tissues including liver, intestine, kidney and brain. The transporters play a crucial role in the absorption, distribution and excretion of xenobiotic (Xu *et al.*, 2005).

Besides toxicity, xenobiotics can also be therapeutic beneficial as in the case of drugs. Thus modification of therapeutic agents by drug-metabolizing enzymes can also change their biological effects. Therefore, drug-metabolizing enzymes play a vital role in determining the intensity and duration of action of drugs and chemical toxicity and chemical tumorigenesis (Klaassen, 2008).

1.2.2 Monofunctional and bifunctional inducers

Although most of the drug-metabolizing enzymes are constitutively expressed, their synthesis can be induced by some xenobiotics. The existence of mono- and bifunctional inducers was proposed by Talalay (Prochaska *et al.*, 1985; Prochaska & Talalay, 1988). Mono-functional inducers selectively increase the expression of genes encoding NQO1 and GSTs that are regulated through the presence of an antioxidant response element (ARE) in their promoter regions. Such induction is mediated by transcription factor, the nuclear factor erythroid 2-related factor 2 (Nrf2), as a consequence of inhibition of its repressor Kelch-like ECH-associated protein 1 (Keap1). Bifunctional inducers increase the expression of NQO1 or GSTs as well as proteins from P450 family, such as CYP1A1. They can bind with high affinity to the aryl hydrocarbon (Ah) receptor and lead to its activation which upon translocation to the nucleus in turn binds to the xenobiotic responsive element (XRE) and transactivates genes responsible for some enzymes in the P450 family such as Cyp1a1, Cyp1a2 and Cyp1b1.

1.2.3 Induction and regulation of Nqo1

Inducers of Nqo1 can be classified into nine structurally diverse classes (Figure 1.8): 1. Oxidizable diphenols, phenylenediamines and quinones; 2. Michael reaction acceptors; 3. Isothiocyanates, dithiocarbamates and related sulfur compounds; 4.

1,2-Dithiole-3-thiones, oxathiolene oxides, and other organosulfur compounds; 5. Hydroperoxide; 6. Trivalent arsenicals; 7. Heavy metals; 8. Vicinal dimercaptans; and 9. Carotenoids and related polyenes. (Dinkova-Kostova *et al.*, 2005; Russo, 2007). Although structurally distinct, these chemicals share common properties of electrophilicity and capacity to modify sulfhydryl groups. Blocking the initiation of tumours by administration of certain of these inducers has been seen in various tissues, such as liver, colon, mammary gland and pancreas (Myzak & Dashwood, 2006). There is substantial and mounting evidence that drug metabolizing enzymes, e.g., N-acetyltransferase (NAT), GST, SULT, and UGT, and NQO1, play important roles in the detoxification of electrophilic toxicants and their induction protects against carcinogenesis and mutagenesis. Induction of certain drug-metabolizing enzymes is regulated at the transcriptional level through AREs in the promoter region of their genes. The transcription factor Nrf2 is principally responsible for mediating basal and inducible expression of ARE-driven genes; Nrf2 binds the ARE as a heterodimer with small Maf proteins. The most compelling evidence that Nrf2 makes a major contribution to the regulation of ARE-driven genes comes from the study of Nrf2 knockout mice. In particular, the basal and inducible expression of Gst and Nqo1 is substantially reduced in *Nrf2*$^{-/-}$ mice when compared with their wild-type counterparts (Itoh *et al.*, 1997; McMahon *et al.*, 2001). Besides Nrf2 and small Maf proteins, other transcription factors may influence ARE-driven gene expression. *In vitro* DNA binding studies using antibody supershift assays have shown that Nrf1 and members of the AP-1 family can bind the ARE (Nguyen *et al.*, 2003). Besides

these transcription factors, several intracellular and signal transduction pathways are involved in the activation of Phase II enzymes. These pathways include the mitogen-activated protein kinase (MAPK) pathway, the protein kinase C (PKC) pathway, and the phosphatidylinositol 3-kinase (PI3K) pathway (Huang *et al.*, 2002; Lee *et al.*, 2001; Yu *et al.*, 1999)

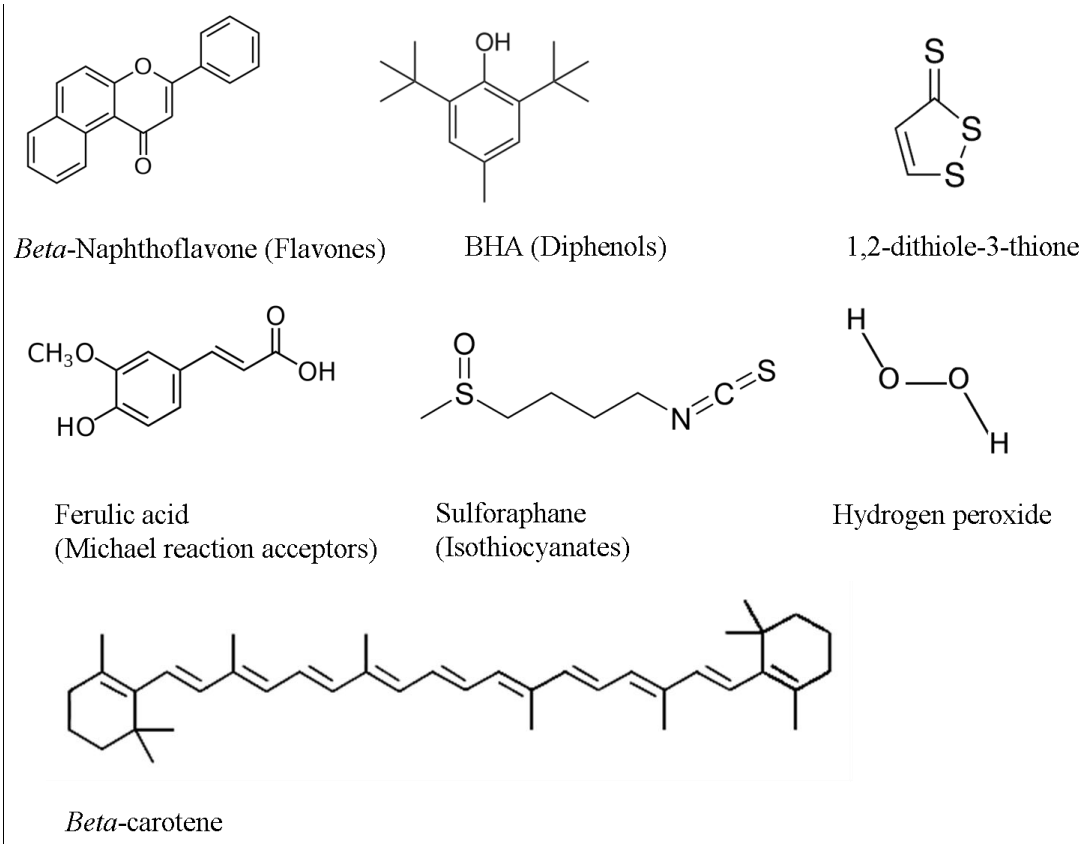


Figure 1.8 Structures of typical Nqo1 inducers.
Typical inducers from each class are selected and presented. The names in the brackets are the classes they belong to.

1.2.4 Antioxidant responsive element

The ARE is a cis-acting enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress. Proteins that are members of the ARE-gene battery include those associated with glutathione biosynthesis, redox

proteins with active sulfydryl moieties and drug-metabolizing enzymes. The cis-acting element was first identified within the 5' flanking region of a 41bp DNA sequence in the rat *GSTA2* gene containing 41-bp DNA and was later designated as the ARE due to its responsiveness to phenolic antioxidants (Rushmore *et al.*, 1991). The core DNA sequence essential for the response to these chemicals was determined through deletion and mutational analysis and was firstly defined as 5'-TGACnnnGC-3' (Favreau & Pickett, 1991). However, the same study also showed that nucleotides situated immediately 5' to the 'core' ARE were also required for basal and inducible expression of the gene. Consistent with this finding, research from another group proposed the extended ARE core sequence as 5'-TMAnnRTGAYnnnnGCRwwww-3' (M=A/C; R-A/G; Y=C/T; and W=A/T), demonstrating the importance of flanking sequence for the context-specific regulation of gene transcription (Wasserman & Fahl, 1997). In 2003, through point mutations across the whole ARE in the mouse Nqo1 promoter, Nioi et al. found that the 3'tetra-nucleotide 'wwww' is required for neither basal nor inducible gene expression. The same study also revealed that nucleotides previously suggested to be redundant (which are shown as 'n' in the sequence mentioned above) are required for gene induction, and on the other hand, the core sequence that had been reported previously to be essential before was found to be dispensable in the case of mouse *Nqo1* (Nioi *et al.*, 2003). Take together, these studies indicated that the sequence of ARE in the promoter of different genes may be distinct. In addition to the genes that encode the rat GSTA2 and mouse Gsta1 proteins, genes encoding the rat and

human NQO1 proteins (Favreau & Pickett, 1991; Jaiswal, 1991), glutamate cysteine ligase catalytic subunit (GCLC) and modulatory (GCLM) subunits (Moinova & Mulcahy, 1998; Mulcahy *et al.*, 1997; Wild *et al.*, 1998), and HO-1 (Inamdar *et al.*, 1996) were also found to be transcriptionally regulated via the ARE. Table 1.3 shows the ARE sequence present in the promoter region of different ARE-driven genes.

Sequence of the ARE in the promoter in various genes

Function	Species	*Gene*	Element	Sequence
Antioxidant enzymes	Human	*GCLC*	ARE-4/AP1	**TC**ccc**GTGAC**tca**GCG**
		GCLM	EpRE	ag**A**ca**ATGAC**taa**GCA**
			ARE (var)	**TAA**cg**GT**t**AC**gaa**GCA**
		GPX2	ARE-1	c**CAggATGAC**tta**GCA**
			ARE-2	gt**A**ca**GTGAg**agg**GCA**
		PRDX1	EpRE-1	**T**gtaac**TGA**atca**GC**c
			EpRE-2	**T**tctcc**TGcC**tca**GC**c
		PRDX6	ARE	g**CA**ac**GTGAC**cga**GC**c
		TRX	ARE/AP1	**TCA**cc**GT**t**AC**tca**GCA**
		TXNRD1	ARE	**TCA**ga**ATGAC**aaa**GCA**
	Mouse	*Gsr1*	ARE-1	**TC**gcc**GTGAC**taa**GCA**
			ARE-2	**TCA**ca**GTGAC**caa**GCG**
		Slc7a11	EpRE-2	c**CA**gct**TGA**gaaa**GCG**
	Rat	*SRXN1*	ARE-1/AP1	**TCA**ccc**TGA**gtca**GCG**
Metal-binding proteins	Human	*FTL*	MARE/ARE	**TCA**gc**ATGAC**tca**GCA**
		MT1B	ARE	g**A**gca**GTGAC**ctg**GCG**
	Mouse	*Fth1*	FER1	c**C**tcc**ATGAC**aaa**GCA**
			AP1/NF-E2	c**CA**cc**GTGAC**tca**GCA**
		Ftl1	EpRE	**TCA**gc**GTGAC**tca**GCA**
		Mt1	ARE	ggcgc**GTGAC**ctg**GC**c
		Mt2	ARE/AP1	ggggt**GTGAC**tca**GCG**
Detoxification proteins	Human	*AKR1C2*	ARE	**TCA**gg**GTGAC**tca**GCA**
		MGST1	EpRE	a**CA**tc**GTGAC**aaa**GCA**
		NQO1	ARE/AP1	**TCA**ca**GTGAC**tca**GCG**
		UGT1A1	ARE	a**AA**ccc**GAC**ttg**GC**c
	Mouse	*Akr1b3*	ARE-1	gg**A**gc**ATGAC**cca**GCA**
		Gsta1	EpRE	**TAA**tg**GTGAC**tca**GCA**
		Gsta3	ARE	c**A**ggc**ATGAC**att**GCA**
		Mrp2	ARE	ctggg**ATGAC**ctc**GCA**
		Nqo1	ARE	**TCA**ca**GTGAg**t**c**g**GCA**
	Rat	*Gsta2*	ARE	**TAA**tg**GTGAC**aaa**GCA**
		Gstp1	GPE1/AP1	**TCA**ct**ATGAT**tca**GCA**
		Nqo1	ARE	**TCA**ca**GTGAC**t**t**g**GCA**
			ARE ´ core´	**TGAC**nnn**GC**
			ARE consensus	**TMA**nn**RTGAY**nnn**GCR**
			Ap1-binding site	**TGAS**tca

Table 1.3 ARE sequences in the promoter of different genes from different species.

The sequences shown are from the genes for antioxidant, metal-binding, and detoxication proteins from human, mouse and rat. As Ap-1binding site share some similarities in the sequence of ARE, the sequence of AP-1 binding site in the genes, and its core sequence were also shown in the table. The nucleotides in bold capital letters are those that share identity with the extended 16-bp ARE consensus sequences (5 ´TMAnnRTGAYnnnGCR´). M=A/C; R=A/G; Y=C/G/T. Data are adapted from Hayes *et al.* (2010)

1.2.5 Induction of drug-metabolizing enzymes and cancer chemoprevention

Carcinogenesis is a complex and protracted multistage process, yet the entire course can be initiated by a single event wherein a cellular macromolecule is damaged by an endogenous or exogenous agent. Strategies for protecting cells from these initiating events include increasing drug-metabolizing enzymes which are involved in promoting the conjugation and excretion to reduce their toxicity. Reduction of electrophilic quinones by NQO1 is an important detoxification pathway, which converts quinones to hydroquinones and reduces oxidative cycling. Therefore, chemicals which can increase the expression or activity of NQO1 may help to prevent the initiation of cancer.

1.2.6 Induction and regulation of P450s

Cytochromes P450 play important roles in drug, carcinogen, and steroid hormone metabolism (Estabrook, 1996). There are 18 CYP mammalian gene families , and four of these families (1, 2, 3, and 4) principally metabolize foreign compounds including drugs, food additives and environmental pollutants (Nebert & Dalton, 2006). Some cytochrome P450 enzymes are substrate inducible, a property that allows the cell to adapt to changes in its chemical environment. Induction of CYP enzymes has both advantage and disadvantages. On one hand, enzyme induction inhibits chemical carcinogenesis because it increases the rate of carcinogen detoxification which will prevent the accumulation of lipophilic compounds to

harmful levels. On the other hand, as cytochrome P450 enzymes have broad substrate specificities, enzyme induction by one compound may lead to increased metabolism of a second compound, producing loss of drug effect. In addition, cytochrome P450 induction can produce an imbalance between detoxification and activation reactions, leading to adverse effects. In the case of polycyclic aromatic hydrocarbons (PAHs), found in cigarette smoke, metabolism by cytochromes P450 can generate arene oxides which are electrophiles that bind covalently to cellular components. At high concentrations, where detoxification pathways may become saturated, induction can increase the production of reactive metabolites beyond the capacity of cellular defences, thereby producing toxicity or neoplasia (Conney, 1982; Weisburger, 1978).

The CYP superfamily contains up to 18 families of mammalian genes and they are regulated through different mechanisms. The induction of the CYP1 family is regulated by a heterodimer composed of the (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt) (Hankinson, 1995), while the expression of CYP2, 3, 4 family members is regulated by the nuclear factor constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor (PPAR), respectively (Kawajiri & Fujii-Kuriyama, 2007). It is also noteworthy that some P450s such as Cyp2c55 andCyp2u1 can also be regulated by Nrf2 (Hu *et al.*, 2006).

1.2.7 Regulation of CYP1A1 through the XRE

Typical inducers of CYP1A1 include halogenated aromatic hydrocarbons (HAH) and polycyclic aromatic hydrocarbons (PAH), and the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Studies into the mechanism of CYP1A1 induction have employed TCDD as the xenobiotic inducer. Understanding the basis of CYP1A1 induction is important because it is involved in the metabolism of PAHs and the production of reactive genotoxic metabolites that may initiate carcinogenesis. Studies using AhR-defective or Arnt-defective cells showed that induction of CYP1A1 is AhR/Arnt dependent (Jones *et al.*, 1986). Later work revealed the protein-DNA interaction between AhR/Arnt complex and the *cis*-element 5'-TNGcGTG-3', which is present in multiple copies within the enhancer of *CYP1A1* (Denison *et al.*, 1988). This element was designated the XRE, but has also been called the dioxin responsive element, or the Ah-responsive element (Whitlock, 1999). In this thesis it is referred to as the XRE. Further investigations using mutational analysis indicated the core sequence essential for the functional XRE is 5'-CGTG-3' (Shen & Whitlock, 1992).

1.3 The CNC-bZIP Nrf2 transcription factor

1.3.1 CNC-bZIP family of transcription factors

The CNC-basic leucine zipper (CNC-bZIP) family, a subfamily of bZIP proteins, play important roles in mammalian development and the regulation of expression of genes involved in various biological processes, including proliferation, apoptosis, differentiation, and stress responses. The first isolated CNC-bZIP protein was the nuclear factor-erythroid 2 p45-subunit (NF-E2 p45) (Chang *et al.*, 1993). Subsequently, another three closely related transcription factors Nrf1 (Chan *et al.*, 1993), Nrf2 (Moi *et al.*, 1994) and Nrf3 (Derjuga *et al.*, 2004) were cloned. In addition, two distantly related proteins were also isolated and named Bach1 and Bach2 (Oyake *et al.*, 1996). The CNC-bZIP transcription factors are composed of two conserved structural domains, named the 'CNC' domain and bZIP domain, with the CNC domain situated just N-terminal to the bZIP domain (Chan *et al.*, 1993; Chan *et al.*, 1998; Moi *et al.*, 1994). Members of the CNC-bZIP family form heterodimers with the bZIP small Maf proteins, composed of MafK, MafF and MafG, to bind to DNA sequences with different specificity (Motohashi *et al.*, 1997). Both the CNC and the bZIP domains are responsible for the DNA binding property and binding specificity of the transcription factors.

1.3.2 Stucture of Nrf2

The CNC bZIP transcription factor Nrf2 contains six domains, namely Neh1-Neh6

(Figure 1.8), which are conserved amongst species (Itoh *et al.*, 1995; Itoh *et al.*, 1999). The Neh1 domain comprises a bZIP region fused to a CNC region and is responsible for its ability to dimerize with small Maf proteins and its ability to bind DNA as an obligate heterodimer. The N-terminal Neh2 domain is required for redox-sensitive negative control of the CNC-bZIP factor (Itoh *et al.*, 1999). The C-terminal Neh3 domain interacts with chromodomain helicase DNA-binding protein 6 (CHD6) and therefore might associate with the transcriptional apparatus (Nioi *et al.*, 2005). Both Neh4 and Neh5 are transactivation domains that interact with CREB-binding protein (CBP) (Katoh *et al.*, 2001). The central Neh6 domain contributes to redox-independent negative control of Nrf2 (McMahon *et al.*, 2004).

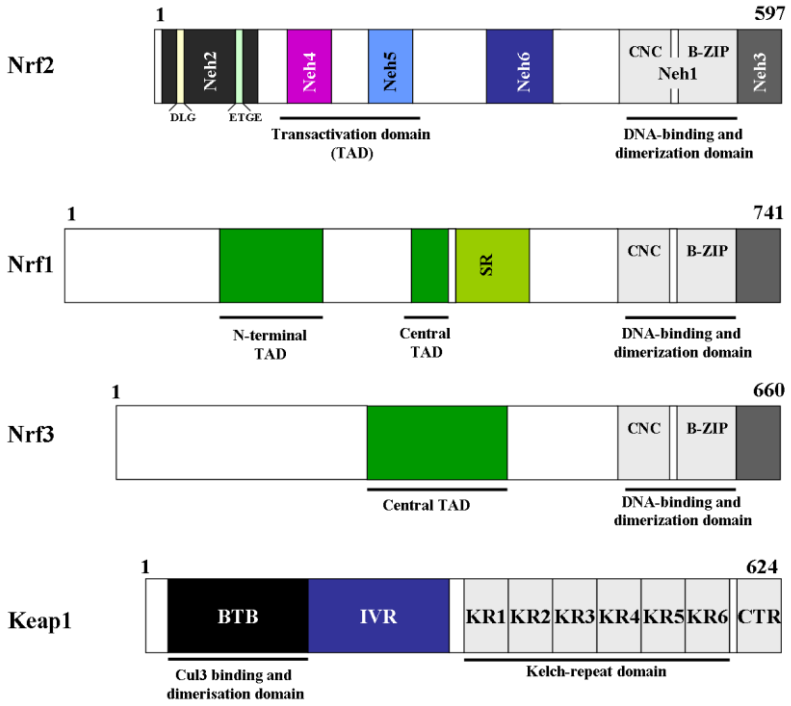


Figure 1.9 Schematic representation of the structural domains of Nrf2, Nrf1, Nrf3 and Keap1. Location of the Neh1-Neh6 domains in rat Nrf2 was shown in the cartoon. The conserved CNC and bZIP domains were compared amongst Nrf1, Nrf2 and Nrf3. Structure of the Keap1, the negative regulator of Nrf2 was also shown. Cartoons were adapted from Hayes & McMahon et al. (2009). CTR, C-terminal region; KR, kelch repeat.

1.3.3 Function of Nrf2 in normal cells

In mammalian cells, Nrf2 enables adaptation to oxidants and electrophiles by stimulating the transcriptional activation of around 100 cytoprotective genes (Hayes & McMahon, 2009) each containing at least one ARE in their promoters (Favreau & Pickett, 1991; Nioi *et al.*, 2003). Genes whose expression is controlled by Nrf2 include those encoding antioxidant proteins, enzymes involved in NADPH regeneration, drug-metabolizing enzymes (Table 1.4), drug-efflux pumps, heat shock proteins, α- and β-subunits of the 26S proteasome, growth factors, growth factor receptors, and various transcription factors (Hayes & McMahon, 2009). The elevated expression of cytoprotective genes can lead to increased capacity of cells to scavenge ROS which can cause lipid, DNA and RNA oxidation that can initiate tumour formation. In addition, the increased level of drug-metabolizing enzymes and drug-efflux pumps enables the detoxification of a wide range of toxic compounds including those containing α,β-unsaturated carbonyl, epoxide, halide, hydroperoxide and quinone moieties, and the removal of their inactive conjugated metabolites from cells (Klaassen, 2008). Overall, the upregulation of ARE-driven genes by activation of Nrf2 enables cells to adapt to increased concentrations of ROS, reactive nitrogen species, and numerous electrophiles. Furthermore, studies using transgenic Nrf2 knock out mice revealed that disruption of *Nrf2* is associated with a marked increased susceptibility to hyperoxia (Cho *et al.*, 2002), and various forms of chronic lung disease produced by exposure to cigarette smoke (Cho *et al.*, 2002). In addition, Nrf2 can also protect against the formation of DNA adducts or mutations produced

by aflatoxin B_1, benzo[a]pyrene and diesel exhaust fumes (Aoki *et al.*, 2007; Aoki *et al.*, 2001; Ramos-Gomez *et al.*, 2003).

Nrf2-regulated drug-metabolizing enzymes from different species		
Species	Gene	Selected inducer
Human	*NQO1*	β-NF, tBHQ
	γ-GCS	BHA
	UGT1A6	t-BHQ
	UGT1A8	EGCG
	UGT1A10	EGCG
Mouse	*GSTA1*	t-BHQ, SFN, 3-MC, catechol
	GSTPi	
	γ-GCS	
	NQO1	BHA, SFN, I3C
	UGT1A6	EQ, OTZ
	UGT2B5	Curcumin
Rat	*Nqo1*	B-NF, t-BHQ
	GSTA	
	GSTPi	
	UGT1A6	EQ, OTZ
	UGT1A7	EQ, OTZ
	UGT2B1	EQ, OTZ
	UGT2B3	EQ, OTZ
	UGT2B12	UGT2B12

Table 1.4 Nrf2 regulated drug-metabolizing enzymes from different species.
Drug metabolizing enzymes from different species which are regulated by Nrf2 are shown in the table. Selected inducers of each drug metabolizing enzymes are also shown (Shen & Kong, 2009).

1.3.4 Negative regulation of Nrf2 by Keap1

The activity of Nrf2 is primarily regulated by Keap1 by binding to its Neh2 domain (Itoh *et al.*, 1999). Nrf2 is a highly unstable protein ($t_{1/2}$ ~15 min), subject to proteolytic degradation catalyzed by the 26S proteasome via the ubiquitin-dependent pathway (Nguyen *et al.*, 2003; Stewart *et al.*, 2003). Studies by different groups showed that the association of Keap1 with Nrf2 promotes Nrf2 ubiquitination in a constitutive manner (McMahon *et al.*, 2003; Zhang & Hannink, 2003) through the cullin 3-dependent pathway (Kobayashi *et al.*, 2004; Zhang *et al.*, 2004). Moreover, study by genetic knockdown of the cellular Keap1 protein or using $Keap1^{-/-}$ animals indicated that upon interaction with Keap1, Nrf2 is targeted directly for

ubiquitination and degradation.

Mammalian KEAP1 proteins are metalloproteins that contain a Broad-complex, Tramtrack, Bric-à-brac (BTB) dimerization domain, an intervening region (IVR) enriched with Cys residues, and a protein docking site, a domain comprising six Kelch repeats (Adams *et al.*, 2000; Dinkova-Kostova *et al.*, 2005; Li *et al.*, 2004; Zipper & Mulcahy, 2002). Most significantly, the cysteines play an important role in regulating the substrate adaptor function of Keap1. Mouse and human KEAP1 proteins contain 25 and 27 cysteines, respectively, around half of which are likely to be highly reactive, able to form thiolate anion under normal physiological conditions (Hayes & McMahon, 2009). These Cys residues thus present Keap1 as an attractive target for potential regulation by thiol-reactive chemical species and, hence, inhibitory modulation of its activity was suggested to be an important mechanism for Nrf2 activation (Dinkova-Kostova *et al.*, 2002; Dinkova-Kostova *et al.*, 2001; Itoh *et al.*, 1999; Wakabayashi *et al.*, 2004; Zhang & Hannink, 2003). Overexpression of recombinant KEAP1 in various cell lines has shown that Cys23, Cys273 and Cys288 (Kobayashi *et al.*, 2004; Nioi & Nguyen, 2007; Zhang & Hannink, 2003) are required for its repression of Nrf2. In addition, Cys151 appeared to be required for inhibition of the substrate adaptor activity of Keap1 by inducing agents (Zhang & Hannink, 2003).

Very recently, a two-site substrate recognition model, also called the hinge and latch

model, was presented to explain how Keap1 recruits Nrf2 and assists in ubiquitination of the CNC-bZIP protein by Cul3-Rbx1 (McMahon *et al.*, 2006; Tong *et al.*, 2006). In this model, each Kelch-repeat domain from a Keap1 homodimer binds to one Nrf2 protein through a weak-binding DLG motif (residues 29-31) or a strong binding ETGE motif (residues 76-84) located in the N-terminal Neh2 domain of Nrf2. The binding affinity of Kelch to the ETGE motif is approximately 100-fold higher than that of Kelch to the DLG motif. Several studies suggest that the Keap1 homodimer binds both the DLG and ETGE motifs in Nrf2 to align the seven ubiquitin-accepting lysine residues between these two motifs into a conformation suitable for ubiquitin conjugation. Upon oxidative stress, modification of the cysteine residues on Keap1, such as C151, C273, or C288 in the BTB or linker domain, imposes a conformational change that disrupts the weak Kelch-DLG binding, resulting in diminished Nrf2 ubiquitination without dissociation of Nrf2 from Keap1. Such dissociation will lead to increased level of Nrf2 protein resulting in the activated Nrf2 signalling pathway (Itoh *et al.*, 1999; Kobayashi *et al.*, 2004; McMahon *et al.*, 2003; Zhang & Hannink, 2003; Zhang *et al.*, 2004).

Another model also exists to explain the stabilization of Nrf2 which is to induce the ubiquitination of Keap1. In this model, the triggering of ubiquitination of Keap1 is by modification of Cys residues in it and it has been reported that treatment with certain xenobiotics can trigger the ubiquitination of Keap1 (Hong *et al.*, 2005; Zhang *et al.*, 2005).

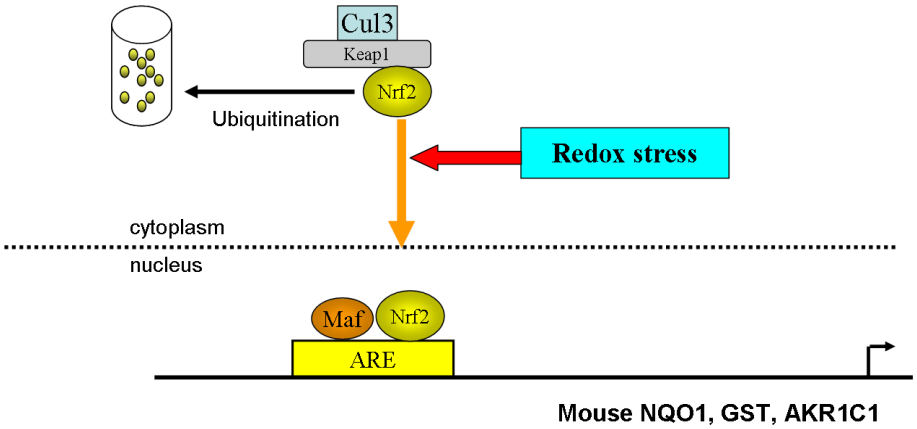


Figure 1.10 Negative regulation of Nrf2 by Keap1 and its activation upon redox stress.
Under homeostatic condition, Nrf2 is targeted for ubiquitination by Keap1. Upon redox stress, the Nrf2 will be released from keap1, translocated into nucleus, heterodimerize with small Maf, and bind to ARE in the gene promoter region, leading to its activation.

1.3.5 Other regulators of Nrf2

As Keap1 targets Nrf2 for ubiquitination, it has been suggested that any mechanism that can disrupt the interaction between the two proteins would lead to the activation of Nrf2. Several protein kinases have been implicated directly or indirectly in the modification of Nrf2, resulting in its activation, including PKC, ERK, MAPK, p38, and PERK. Upon oxidative stress, phosphorylation of Nrf2 at serine 40 by PKC has been reported to release it from Keap1 (Huang *et al.*, 2002). A study by Cullinan *et al.* also suggested that PERK-dependent phosphorylation followed by endoplasmic reticulum (ER) stress triggers dissociation of Nrf2/Keap1 complexes and inhibits re-association of Nrf2/Keap1 complexes in vitro (Cullinan *et al.*, 2003). Besides, activation of several upstream kinases, such as extracelluar signal-regulated kinases 2 and 5 (ERK2 and ERK5), c-Jun NH_2-terminal kinase 1 (JNK1) can lead to the phosphorylation of Nrf2 resulting in its transcriptional activation (Xu *et al.*, 2006). Recently, a research group found that several MAPKs including p38, JNK1/2 and

ERK2 could phosphorylate Nrf2 at Serine 215, 408, 558, 577 and T559. However, such phosphorylation only affect the activity of Nrf2 moderately. Therefore, the author proposed that direct phosphorylations of Nrf2 contribute only a little to the regulation of Nrf2 activity (Sun *et al.*, 2009).

A recent study showed that in response to oxidative stress, p21 is regulated and the ^{154}KRR motif in p21 directly interacts with the DLG and ETGE motifs in Nrf2 and thus competes with Keap1 for Nrf2 binding, compromising ubiquitination of Nrf2. In addition, using p21-deficient mice, this study also demonstrated that p21 mediated upregulation of Nrf2 protein under both basal and induced conditions (Chen *et al.*, 2009). On the other hand, glycogen synthase kinase-3β (GSK3β) negatively regulates Nrf2 signalling via phosphorylation of Nrf2 at tyrosine or serine residues. Furthermore, the p38 MAP Kinase can phosphorylate Nrf2 protein, causing an increased interaction between Nrf2 and Keap1 which in turn attenuates constitutive and inducible Nrf2 activity (Keum *et al.*, 2006; Xu *et al.*, 2006).

1.3.6 Nrf2 gene induction

Besides its regulation by Keap1, ubiquitination and various signalling pathway, there is also possibility that Nrf2 may be regulated at transcriptional level on the basis that the Nrf2 promoter contains two ARE sequences, -579 5´-TGACTCCGC-3´ and -317 5´-TGACTCCGC-3´ (Kwak *et al.*, 2003) and also XRE or XRE-like sequences -712 5´-GCGTG-3´, 755 5´-CACGC-3´, and 870 5´-CACGC-3´(Miao *et al.*, 2005). It has

been shown that Nrf2 mRNA is modestly increased in mouse keratinocytes by 3H-1,2-dithiole-3-thione treatment (Kwak *et al.*, 2003) and the isothiocyanate sulforaphane. Regarding to the presence of XRE in the promoter region, the mRNA of Nrf2 was shown to be increased by TCDD in mouse hepatoma 1c1c7 cells (Miao *et al.*, 2005). Furthermore, multiple single nucleotide polymorphisms exist in the promoter of human *NRF2*, and one of these (-617 C/A) significantly reduces gene expression (Marzec *et al.*, 2007). However, it is not known whether such polymorphisms prevent the variant allele from being transcriptionally activated by thiol-active agents.

1.3.7 Nrf2 in cancer promotion and drug resistance

In the previous sections the beneficial sides of Nrf2 were described; however, this bZIP transcription factor also possesses potentially harmful activities. Several studies have shown that Nrf2 can promote tumourigenesis and chemoresistance. The first evidence suggesting that Nrf2 is involved in cancer promotion was from the finding that Nrf2 and GSTP1 were upregulated during development of heptatocellular carcinoma (Ikeda *et al.*, 2004). Later studies identified the existence of the Keap1 mutation or loss of heterozygosity in the Keap1 locus in lung cancer cell lines or cancer tissues. The ultimate result of Keap1 mutation is the increase Nrf2 activity and transactivation of its downstream genes (Padmanabhan *et al.*, 2006; Singh *et al.*, 2006). An investigation in 65 Japanese patients with lung cancer suggested that there was a high incidence of Keap1 somatic mutations with lung adenocarcinoma (Ohta *et*

al., 2008). Consistently, another report indicated that Keap1 expression was reduced in lung cancer cell lines and tissues, compared to that in normal bronchial epithelial cell line (Wang *et al.*, 2008). Moreover, Nrf2 was found to be overexpressed at later stage of cancer in lung tissue (Wang *et al.*, 2008). In addition, a mutation of Keap1 (C23Y), leading to its inability to repress Nrf2, was found in breast cancer (Nioi & Nguyen, 2007).

Collectively these results suggest that loss of function of Keap1 may result in prolonged activation of Nrf2 prompting the survival of cancer cells. Such consequence may be due to the up-regulation of some of the downstream genes regulated by Nrf2 which can prevent cells from apoptosis.

Besides cancer promotion, Nrf2 also contributes to the resistance of cancer cells to chemotherapy which was indicated in a study showing that prognosis in patients with lung cancer that contain mutant Keap1 or Nrf2 was worse than that in patients with lung tumours lacking such mutations (Shibata *et al.*, 2008). As activation of Nrf2 protects normal cells against cytotoxic agents, it is possible that they protect malignant cells in human tumours against chemotherapeutic drugs. Thus the *in vitro* study by Wang *et al.* investigated the role of Nrf2 in determining drug response in lung carcinoma, breast adenocarcinoma and neuroblastoma. The results showed that up regulation of Nrf2 enhanced cells resistance, whereas down regulation sensitizes cells to chemotherapeutic agents (capsulation, doxorubicin and etoposide) (Wang *et*

al., 2008). Therefore, strategies to overcome drug resistance caused by upregulation of Nrf2 are desirable. Hayes, *et al.* have reviewed the means to solve this problem, either by antagonizing Nrf2 directly or by exploiting upregulated ARE-drive genes to activate cytotoxic pro-drugs (Hayes & McMahon, 2009).

1.3.8 Nrf2 and other diseases

In addition to cancer prevention, activation of Nrf2 and its downstream ARE-driven genes may also prevent neurodegenerative disease (Chen *et al.*, 2009; Shih *et al.*, 2005), neovascular disease (Cano *et al.*), cardiovascular disease (Li *et al.*, 2009) and diabetes. It is noteworthy that common factor in the pathogenesis of all these diseases is oxidative stress. Many studies have proved that the protection exerted by up-regulation of Nrf2 is ultimately due to the increased expression of antioxidant response, which can combat the oxidative insults, followed by activation of Nrf2.

1.4 The aryl hydrocarbon receptor

1.4.1 The structure of AhR protein

The Aryl hydrocarbon receptor (AhR) belongs to the eukaryotic Per-ARNT-Sim (PAS) domain protein family that function as sensors of extracellular signals and environmental stresses affecting growth and development (Gu *et al.*, 2003). Amongst them, AhR regulates adaptive and toxic responses to a variety of chemical pollutants,

including polycyclic aromatic hydrocarbons and polychlorinated dioxins with TCDD as a classic inducer of AhR. The AhR cDNA was first cloned from mouse in 1992 (Burbach *et al.*, 1992; Ema *et al.*, 1992). Subsequently, the human (Dolwick *et al.*, 1993) and rat (Carver *et al.*, 1994) AhR cDNA were also cloned. Though the cDNA of AhR has been cloned in other species such as birds, fish, amphibians, studies on the rodent and human AhR have been the most extensive (Hahn, 2002). In addition, comparative study by Hahn suggested that AhR is highly evolutionarily conserved. Structural analysis of the AhR cDNA in the early study revealed that AhR protein has two structural domains, the basic helix-loop-helix (bHLH) and PAS domains in the N-terminal half of the molecule (Burbach *et al.*, 1992; Ema *et al.*, 1992). The bHLH region contributes to DNA binding and the HLH region to protein-protein dimerization; a nuclear localization signal is contained in this region while nuclear export signals are present in both bHLH and PAS domain. The PAS domain is sub-divided into PAS A and PAS B domains. A study using the yeast Gal4 fusion system provided evidence that the C termini of the AhR harbours a potent transactivation domain (TAD) consisting of proline/serine/thereonine-rich (P/S/T), glutamine (Q-rich) and acidic subdomains, each of which function independently and exhibits varying levels of activation (Jain *et al.*, 1994; Korkalainen *et al.*, 2000; Rowlands *et al.*, 1996). In addition, the AhR shares structural similarity with its nucleus dimerization partner Arnt and its repressor AhRR. Structures of these three proteins are schematically presented in Figure 1.11.

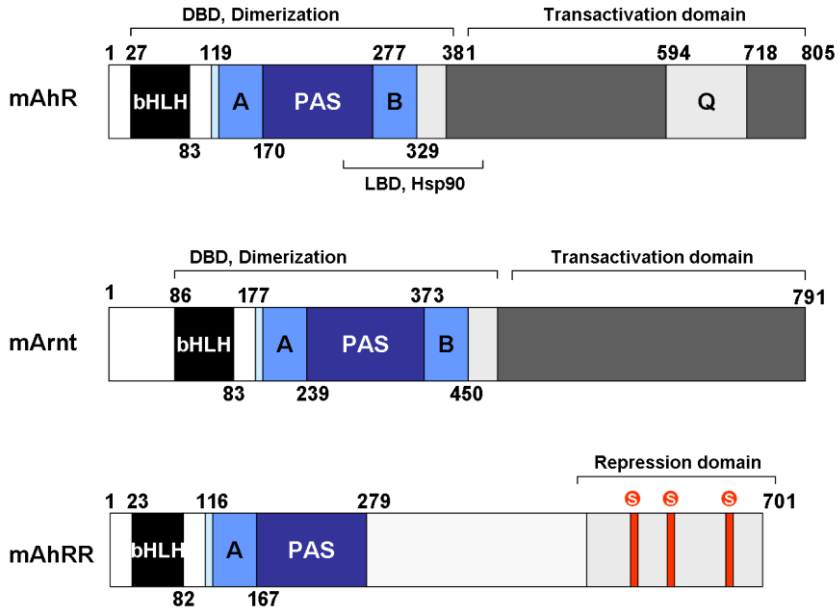


Figure 1.11 Schematic representation of the full-length of mouse AhR, Arnt and AhRR.
The characterized domains represented are the basic helix-loop-helix (bHLH), Per-Arnt-sim (PAS), transactivation or repression domain. Figures are adapted from Fuji-kuriyama & Kawajiri (2010)

1.4.2 Transformation of AhR

The AhR is a soluble ligand-activated transcription factor that is held in the cytoplasm as an inactive protein in a complex with the chaperones Heat shock proteins HSP90, HSP23, and an immunophilin-like protein XAP and p23. HSP90 binding is essential to retain AhR in the cytoplasm and this interaction is thought to mask the nuclear localization signal of AhR. Upon ligand binding, HSP90-bound AhR translocates into the nucleus, and this is followed by dissociation of AhR from the HSP90 complex, whereupon it heterodimerizes with Arnt, another bHLH-PAS protein. This heterodimer subsequently binds to XREs, in the regulatory region of target genes (Hankinson, 1995). A number of other coactivators and various components have been reported to form the transcription complex on the AhR/ Arnt heterodimer, and the interaction and order of the complex formation still needs to be

fully elucidated (Fujii-Kuriyama & Kawajiri, 2010).

After ligand binding, phosphorylation of both AhR itself and the HSP90 complex on several sites are required for the transformation of AhR to the fully functional receptor (Puga *et al.*, 2009). Subsequently, the fully functional AhR can induce the expression of many detoxification genes which have XRE in their promoter region. These genes (table 1.5) include cytochromes P450 such as *CYP1A1*, *CYP1A2*, *CYP1B1*, and *CYP2S1*, and other drug-metabolizing enzymes such as *UGT1A6*, *NQO1*, and ALDH3A1, along with several *GST* isoenzymes (Puga *et al.*, 2009).

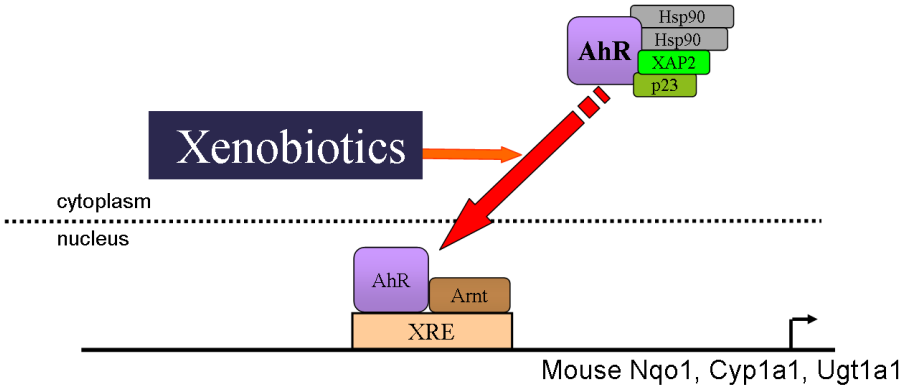


Figure 1.12 Activation of AhR

AhR is kept inactive in cytoplasm by binding to a complex of proteins. Upon ligand binding, it will be released from that complex and translocated into nucleus, where it heterodimerizes with Arnt. The dimer will subsequently bind to XRE in the promoter region of genes and lead to the gene activation.

Drug-metablizing enzymes that are regulated by AhR through XRE	
Species	Gene
Mouse	*Cyp1a1*
	Cyp1a2
	Cyp1b1
Rat	*Cyp1a1*
	Aldh3a1
	Ugt1a1
	Ya subunit of GST
	Nqo1

Table 1.5 Genes encoding for the drug-metabolizing enzyme that are regulated by AhR
Gene encoding drug-metabolizing enzymes in mouse and rat that are regulated by AhR are summarized in the table (Beischlag *et al.*, 2008; Sun *et al.*, 2004). However, the list of such genes is still growing.

1.4.3 Down regulation of AhR activity

AhR activity can be down regulated either before or after its activation by different mechanisms, including proteasomal degradation of AhR, competitive inhibition of AhR by the AhR repressor (AhRR) and binding to its antagonists.

It was found by *in vitro* experiment that AhR can be rapidly depleted after exposure to its ligand. This event most likely occurs after the activation of its target genes and can be blocked by the proteasome inhibitor MG-132. Such degradation can occur in both cytoplasm and nucleus (Davarinos & Pollenz, 1999; Lees *et al.*, 2003; Pollenz & Dougherty, 2005). Besides proteasomes, degradation of AhR by itself can occur after the receptor translocates into the nucleus and forms an E3 ubiquitin ligase complex with Cullin-4B (CUL4B), damaged-DNA-binding 1 (DDB1), ransducin

β-like 3 (TBL3) and ring box protein 1 (Rbx1). This ubiquitin ligase complex can then catalyze the ubiquitination of several proteins including estrogen receptor (ER) α and β, androgen receptor (AR) and AhR itself (Ohtake *et al.*, 2007).

In addition, a negative feedback exists in AhR signalling implying that AhRR which can be transcriptionally induced by activated AhR (Mimura *et al.*, 1999). The promoter region of AhRR contains a functional XRE sequence enabling the activation of AhRR gene expression upon ligand activation of AhR. As AhRR contains a bHLH-PASA region that is structurally similar with AhR, followed by a C-terminal transcription repression domain, AhRR also forms a heterodimer with Arnt (Mimura *et al.*, 1999). This heterodimer binds competitively to the XRE sequence with the AhR/Arnt heterodimer and subsequently recruits co-repressors (Oshima *et al.*, 2007). Therefore, the activation of AhRR ultimately leads to the inhibition of AhR (Baba *et al.*, 2001; Mimura *et al.*, 1999).

1.4.4 Ligands of AhR

AhR ligands include exogenous and endogenous compounds and exhibit structural diversity, though their binding affinities differ to a great extent. Exogenous ligands consist of not only synthetic xenobiotics but also normal dietary components. Amongst AhR ligands that have been identified and characterized, exogenous synthetic ones which show the highest affinity which include planar, hydrophobic HAHs (e.g. polyhalogeneated dibenzo-p-dioxins, dibenzofurans, and biphenyls) and

PAHs (such as 3-methylcholanthrene, benzo(a)pyrene, benzanthracenes, and benzoflavones). Between HAHs and PAHs, the former is more metabolically stable and act as the most potent class of AhR ligands, with binding affinities in the pM to nM range, whereas PAHs, the more metabolically labile ones, bind with relatively lower affinity in the nM to μM range (Denison & Nagy, 2003).

Dietary chemicals acting as ligands of AhR have been described in numerous studies which showed that those chemicals can either activate or inhibit the AhR signalling pathway. In 1978, Watternberg and Loub showed that indoles occurring in edible cruciferous vegetables can inhibit the formation of neoplasia induced by AhR in mice indicating they can inhibit the activity of AhR (Wattenberg & Loub, 1978). However, in 1991, another group showed that one of the indoles Indole-3-carbinol (I3C) can increases the CYP1A1 activity and act as an AhR agonist (Bjeldanes *et al.*, 1991). Besides I3C, more dietary plant compounds have been reported to competitively bind to the AhR, such as curcumin (Ciolino *et al.*, 1998), quercetin and kaempferol (Ciolino *et al.*, 1999). On the other hand, some dietary plant chemicals have been identified as inhibitors of AhR, such as resveratrol (Ciolino *et al.*, 1998). It is noteworthy that many dietary chemicals themselves have little or no AhR ligand activity; however, once they entered the mammalian digestive tract, they may undergo conversion and become significantly more potent AhR ligands. Examples of such chemicals include I3C which itself is a weak inducer of gene expression, whereas, ICZ, an acidic condensation product formed from I3C has very high affinity

of AhR (~0.2-3.6 nM) (Denison & Nagy, 2003).

In addition to exogenous ligands, a number of endogenous ligands of AhR also exist. Evidence for the presence of endogenous ligands has been found in various studies. Firstly, the identification of nuclear AhR complexes in unexposed cells in culture and tissue slices indicated the existence of endogenous ligands. Secondly, the fact that AhR deficient cells had altered cell cycle progression (Ma & Whitlock, 1996; Wei *et al.*, 1996) suggested the effect of endogenous ligands. Thirdly, the AhR knockout animals exhibited numerous physiological changes and developmental abnormalities suggesting AhR can be activated by endogenous ligands (Lahvis *et al.*, 2000; Schmidt *et al.*, 1996). A number of the candidates for endogenous ligands of AhR, bearing various structures have been suggested. These include: indigoids, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), equilenin, arachidonic acid metabolites, haem metabolites, tryptophan metabolites, and ultraviolet photoproducts of tryptophan (Nguyen & Bradfield, 2007).

Taken together, these studies indicate that AhR can bind many different chemicals including environmental contaminants, therapeutics, naturally occurring chemicals and small molecules isolated from tissues. These chemicals have diverse structures and can bind to the AhR with various affinities.

1.4.5 Physiological functions of AhR

Some of the AhR inducers as discussed before are PAHs, PCBs, and dioxins. They are environmental pollutants causing acute and chronic toxicity and some of them are carcinogens. A number of them induce the expression of genes for xenobiotic-metabolizing enzymes such as P450 through the AhR. Besides its involvement in metabolism of xenobiotics, AhR plays a crucial role in various physiological processes (Henklová *et al.*, 2008). Constitutive AhR levels are generally high in liver but AhR is also abundant in diverse mammalian tissues such as placenta, thymus, lung, kidney, small intestine, heart and pancreas (Harper *et al.*, 2006). They have various biological functions ranging from reproduction, developmental function, immunity, cell cycle and cell proliferation, cell adhesion and migration (Barouki *et al.*, 2007).

Indications that AhR is involved in normal physiological processes came from studies showing that AhR can be activated in a xenobiotic-independent way (Allan & Sherr, 2005; Chang & Puga, 1998; Richter *et al.*, 2001; Singh *et al.*, 1996). Furthermore, AhR-null mice provided a deeper insight into the physiological process requiring AhR activity. These animal models not only demonstrated that this receptor is essential for dioxin-induced toxicity (Fernandez-Salguero *et al.*, 1996) and carcinogenesis (Shimizu *et al.*, 2000), but they also revealed the existence of an AhR-deficient phenotype. Different studies showed that genetic deletion of the AhR in mouse caused early death or pathological changes by 13 months and was

accompanied by a wide variety of phenotypic alteration in major organ systems including cardiovascular system, characterized by progressive cardiac hypertrophy, gastric hyperplasia that progressed into polyps with age, T cell deficiency in spleens, and abnormalities in skin such as hyperkeratosis, and marked dermal fibrosis (Fernandez-Salguero *et al.*, 1997; Lund *et al.*, 2006).

Besides its effect on the cardiovascular system, the immune system and skin, AhR-null females showed difficulties in maintaining pregnancy and their pups showed poor survival during lactation and weaning (Abbott *et al.*, 1999). In addition, AhR has a significant impact on the development of liver. The livers of AhR null mice are smaller in size and show portal fibrosis and early lipid accumulation (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). Comparing liver mRNA profiles between wild- type and AhR-null mice showed that the expression patterns of 392 genes were changed due to the absence of AhR. The mechanisms that underlie these physiological functions of AhR include its effect on the cell cycle which in turn can affect the progress of cell proliferation, either inhibiting or promoting it depending on the cell phenotype (Barouki *et al.*, 2007) which will be discussed later. The AhR is also involved in developmental processes and cell adhesion and migration (Barouki *et al.*, 2007).

1.4.6 Involvement of AhR in the progress of tumourigenesis

As the AhR can both promote and inhibit cell proliferation, there has been some

discussion about whether it is a tumour promoter or tumour suppressor.

The AhR cooperates with signalling molecules involved in survival pathways that sustain cell proliferation. An example is NF-κB, with which AhR can physically interact, leading to its activation in human breast cancer MCF-7 cells. Activation of NF-κB causes the transactivation of the c-myc proto-oncongene, thus by this mechanism the AhR may contribute to increased proliferation and carcinogenesis in the breast (Dong *et al.*, 2000). Another study also showed that AhR can induce cell proliferation in human lung carcinoma A549 cells due to the over-expression of the receptor. Mice expressing a constitutively active AhR also showed increased frequency of the formation of induced hepatocarcinomas by N-ntrosodiethyl (Moennikes *et al.*, 2004) and spontaneous tumours in the glandular stomach (Andersson *et al.*, 2002). As activation or over-expression of AhR can stimulate cell proliferation and even carcinogenesis, the receptor appears to have oncogenic activity.

On the other hand, several studies found that activation of AhR can halt the cell cycle at different stages and thus inhibit cell proliferation. In non-proliferating 5L-heptoma cells, activation of AhR by exogenous ligands can trancriptionally activate the tumour suppressor $p27^{kip1}$. Consistent with this observation was that induction of $p27^{kip1}$ by dioxin in fatal thymus was accompanied by inhibition of cell proliferation (Kolluri *et al.*, 1999). As mentioned earlier, the AhR can stimulate cell proliferation

in MCF-7 cells without exogenous ligand. By contrast, in the presence of exogenous ligands, AhR can synergize and interact with the pRb tumour suppresser resulting in the inhibition of pRb-mediated E2F-dependent transcription and ultimately leading to cell cycle arrest (Puga *et al.*, 2000). Another study also showed that cell cycle was blocked at G_1 by dioxin in MCF-7 and mouse hepatoma Hepa-1 cells. Such blockage was due to the interaction between activated AhR and the p300 co-activator, leading to displacement of p300 from E2F-dependent promoter and proliferation arrest (Marlowe *et al.*, 2004). Cell cycle arrest by constitutively activated AhR has also been found in a few other cell lines, transgenic mice, and mouse thymus in organ culture through different mechanism (Barouki *et al.*, 2007). Taken together, these findings suggest that constitutive or induced activation of AhR by exogenous ligands may act like a tumour suppressor by inhibiting cell proliferation.

Therefore, depending on the phenotype of cells and inducers, AhR can either inhibit or promote cell proliferation and thus have tumour suppressor or oncogenic activity.

1.4.7 Cross talk between AhR and other signalling pathways

As discussed earlier, besides mediated dioxin-induced toxicity, AhR also influences many physiological functions. The mechanisms that underlie the wide diversity of AhR activity is its cross talk with multiple signalling transduction pathways including mitogen-activated protein kinases (MAPKs), cell cycle progression and apoptosis, and transcriptional factors such as hypoxia induced factor (HIF1) and

Nrf2.

The MAPKs, mediating important intracellular transduction, include three families: extracellular signal kinases (ERK1/2), c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK), and the p38s. MAPKs or their down-stream MAPK-activated protein kinases can phosphorylate a large panel of substrates on serine and threonine residues, which enable them to regulate gene expression and protein functions (Cobb & Goldsmith, 2000).

In general, ERK1 and 2 are involved in regulating mitogenic and developmental events and the four p38 kinases isoforms play important roles in the inflammatory response, apoptosis and the cell cycle. The three JNK isoforms play important roles in cellular signalling, the immune system, stress-induced and developmentally programmed apoptosis, carcinogenesis and in the pathogenesis of diabetes (Weston & Davis, 2002).

Although TCDD, the well known AhR ligand, activates ERK and JNK, such activation occurred equally in both AhR-positive and AhR-negative cells, suggesting induction of MAPK by AhR ligand in an AhR-independent manner. However, TCDD-stimulated MAPKs appear critical for the induction of AhR-dependent gene transcription and CYP1A1 expression (Tan *et al.*, 2002). A recent study has shown that, in MCF-7 cells, TCDD and another ligand 3-methylcholanthrene induced

morphological changes that modulate epithelial cell plasticity in MCF-7 cells. Such dioxin-induced events were mimicked by constitutive expression and activation of AhR. In addition, a correlated event was the activation of JNK which is reversible using a JNK inhibitor indicating the effects of AhR on cell plasticity is in a JNK dependent pathway. Therefore, these novel effects on cell plasticity support a mechanistic role for the AhR in cancer progression as mediated by many of its ligands (Diry *et al.*, 2006).

Activation of p38 by AhR ligands seems to be a cell-specific consequence. For example, p38 can be activated by TCDD in RAW 264.7 murine macrophages in an AhR-independent mechanism (Park *et al.*, 2005) but not in mouse embryonic fibroblasts.

Another signalling pathway AhR interacts with is the RB-E2F axis which is responsible for several cell cycle check points such as G_1 and S check points. Direct interactions have been found between ligand activated AhR and either the hypophosphorylated RB protein or E2F. Interaction between AhR and RB blocks the phosphorylation of RB leading to the repression of S-phase specific gene transcription. Alternatively, AhR activation can induce CDK inhibitors that arrest the cell cycle in G_1. In addition, one study using $Ahr^{+/+}$ and $Ahr^{-/-}$ fibroblasts showed that the proliferation rate is faster in $Ahr^{+/+}$ fibroblasts compared with that in $Ahr^{-/-}$ fibroblast in a ligand independent manner. Growth-promoting genes were

significantly down-regulated in *Ahr*$^{-/-}$ fibroblast, whereas growth-arresting genes were up-regulated. These results suggested that the Ah receptor may play an intrinsic role in regulating cell proliferation independent of either exogenous of endogenous ligands (Chang *et al.*, 2007). In contrast, AhR-dependent promotion of cell proliferation through induction of JUN-D and cyclin A was also observed (Andrysík *et al.*, 2007).

Activation of E2F1 can activate apoptosis and there is evidence suggesting that E2F1 can act as a tumour suppressor due to its ability to initiate apoptosis in cells that lose normal cell cycle control (DeGregori & Johnson, 2006). It has been found that AhR and E2F1 can physically interact both *in vitro* and *in vivo* resulting in the repression of the transcriptional activity of E2F, and ultimately the inhibition of apoptosis.

Besides physical interaction, AhR can modulate several genes which contain the XRE sequences in their promoter region at the transcriptional level. For instance, the promoter region erythoropoietin (*Epo*), which is transcriptional regulated by HIF1α, contains five functional XRE which can be bound by AhR/Arnt complex and regulate the gene expression of *Epo* (Chan *et al.*, 1999). In addition, AhR has been shown to be able to bind directly to the promoter region of Nrf2 through the XRE sequence (Miao *et al.*, 2005).

1.5 Aim of this thesis

Initially, we carried out screening on a panel of flavonoids using the human mammary AREc32 reporter cell line. Quercetin and kaempferol were found the potent inducers of ARE-driven gene expression. Subsequently, the mechanisms by which these two flavonoids exert their chemopreventive effects, presumably through Nrf2/ARE and/or AhR/XRE, was examined. Experiments were therefore carried out to:

1. Identify whether flavonoids can induce cytoprotective genes.
2. Determine whether flavonoids activate ARE-driven gene expression and whether this involves induction of Nrf2 or inactivation of Keap1.
3. Determine whether flavonoids activate XRE-driven gene expression and whether this involves ligand activation of AhR.
4. Determine whether cross talk occurs between Nrf2 and AhR.
5. Examine whether flavonoids can confer protection against genotoxic compounds.

2 Materials and Methods

2.1 Chemicals and reagent

2.1.1 Chemicals

All chemicals were of the analytic grade, All chemicals were of the highest quality grade and readily available commercially. Dimethyl sulfoxide (DMSO), SUL (>98% pure), 3-MC, 4'-6-diamidino-2-phenylindole (DAPI), 2-nitrophenyl β-D-galactopyranoside (β-gal), 4-hydroxycinnamic acid (p-Coumaric acid), 3-aminophytahydroaxide (luminol), cycloheximide (CHX) and digitonin were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). NADPH was supplied by Melford laboratories Ltd. (Ipswich, UK). All polyphones were obtained from Extrasynthese (Genay Cedex, France). (Table 2.1)

Family	Name	Formula	MW
Flavonoid	Quercetin	$C_{15}H_{10}O_7$	302.07
	Quercitrin	$C_{21}H_{20}O_{11}$	448.38
	Quercetin-3-O-glucopyranoside	$C_{21}H_{20}O_{12}$	464.38
	Rutin	$C_{27}H_{30}O_{16}$	610.53
	Kaempferol	$C_{15}H_{10}O_6$	286.25
	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	448.38
	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	594.53
	Naringenin	$C_{15}H_{12}O_5$	272.27
	Naringenin-7-O-glucoside	$C_{21}H_{22}O_{10}$	434.4
	Hesperidin	$C_{28}H_{34}O_{15}$	610.57
Anthocyans	Cyanidin chloride	$C_{15}H_{11}O_6Cl$	322.7
	Kuromanin chloride	$C_{21}H_{21}O_{11}Cl$	484.82
	keracyanin chloride	$C_{27}H_{31}O_{15}Cl$	630.98
	Myrtillin chloride	$C_{21}H_{21}O_{12}Cl$	500.9
	Oenin chloride	$C_{23}H_{25}O_{12}Cl$	528.87
	Callistephin chloride	$C_{21}H_{21}ClO_{10}$	468.84
Catechin tannis	(-)-Epicatechin	$C_{15}H_{14}O_6$	290.28
	(-)-Epicathein gallate	$C_{22}H_{18}O_{10}$	442.37
	Procyanidin B1	$C_{30}H_{26}O_{12}$	578.53

Table 2.1 Name, formula and molecular weight of different phytochemicals.

2.1.2 Antibodies

NQO1, AKR1C1 and GST antibodies, raised in rabbit were from the Hayes Lab. Rabbit anti-Nrf2 antibody was generously provided by Dr. Michael McMahon. Monoclonal CYP1A1 antibody was a gift from Dr. Colin Henderson (BRI, University of Dundee). Polyclonal antibody against AhR (M-19 and M-20), protein A/G plus-agarose immunoprecipitation reagent (sc-2003), normal goat IgG (sc-2028) and mouse monoclonal Anti-actin antibody were purchased from Santa Cruz

Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal antibody against AhR was purchased from Abcam PLC. (Cambridge, UK). Alexa-Fluor 488 (FITC, fluorescein isothiocyante)-Goat anti-rabbit IgG and Alexa-Fluor 594 (Rhodamine) donkey anti-sheep IgG were from Invitrogen Ltd. (Paisley, UK). Horseradish peroxidase-conjugated secondary antibodies against the mouse or rabbit IgG were purchased from Bio-Rad laboratories. The antibody against glyceraldehye-3-phosphate dehydrogenase (GAPDH) was from Amino Bioproducts Ltd. (Frellstedt, Germany). Lamin A/C was from Upstate Co. (Dundee, UK).

2.1.3 Enzymes

Restriction enzymes were obtained from Promega Corp. (Madison, WI, USA), New England Biolabs (Ipswich, MA, USA) and F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Klenow enzyme, T4 DNA ligase and deoxynucleotide triphosphates (dNTPs) were bought from Promega (Southampton, UK). DNA polymerase was obtained from Stratagene (La Jolla, CA, USA). Reverse transcriptase was from Qiagen (Crawley, UK).

2.1.4 Oligonucleotide primers and DNA plasmids

All oligonucleotide primers for PCR amplification were synthesized by MWG Biotech CO. (Ebersberg, Germany). TaqMan® Gene Expression Assays (Table 2) and TaqMan® Master Mix were supplied by Applied Biosystems (Foster City, CA,

USA). All DNA plasmids were obtained from Invitrogen or Promega.

2.1.5 Cell line, cell culture media and reagents

Name, species and tissue origins of each cell line are listed in table 2.2.

Dulbecco's modification of Eagle's medium (DMEM), Isocoves modified Dulbecco's medium (IMDM), Opti-MEM medium, heat-inactivated foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, insulin-transferin-selenium (ITS), human epidermal growth factor (EGF) and trypsin, were all purchased from Life Technologies Ltd (Paisley, Scotland, UK).

Name	Species	Tissue origins	Cell type
AREc32	*Homo Sapiens*	Mammary gland	Epithelial
RL-34	*Rattus norvegicus*	Liver	Epithelial
MEF	*Mus musculus*	Embryo	Fibroblast
COS1	*Cercopithecus aethipos*	Kidney	Epithelial
HEC116	*Homo Sapiens*	Endometrium	Epithelial
HT29	*Homo Sapiens*	Colon	Epithelial
Caco-2	*Homo sapiens*	Colon	Epithelial

Table 2.2 Name, species, tissue origins and cell type of all the cell lines used in this study.

2.1.6 Animals

7 week old C57BL/6J male mice were purchased from Charles River laboratories (Charles River UK Ltd. Manston Road, Margate, Kent, England)

2.1.7 Other chemicals and reagents

Bradford assay reagent, Bio-Rad *DC* protein assay kit, polyacrylamide and agarose gel kits were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK). Protein molecular mass standards, DNA size ladders for electrophoresis and Lipofectamine™ 2000 were purchased from Invitrogen Ltd. (Paisley, UK). Immobilon-P membrane was obtained from Millipore (Watford, UK). Steady-Glo® Luciferase Assay System and Luciferase Reporter Assay System were from Promega. BCA Protein Assay Reagent (bicinchoninic acid) was from Thermo Scientific Inc. (Rockford, IL, USA).

2.2 Molecular Biology Methods

2.2.1 Basic methods for DNA manipulation

2.2.1.1 Agarose-gel electrophoresis

DNA was resolved on the basis of size by agarose-gel electrophoresis. Gels contained 0.8 – 1.2 % (w/v) agarose with 0.3 µg/ml ethidium bromide in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.0). Samples were mixed with DNA sample buffer (12% (v/v) glycerol, 60 mM EDTA, 0.6 % (w/v) SDS, 0.003% bromophenol blue) and loaded onto gels, alongside size markers (Invitrogen), before being subjected to electrophoresis at 70 V for 60 minutes. DNA was visualized on a

UV translluminator at 320 nm.

2.2.1.2 Ligation

Plasmid vector and insert DNA molecules were ligated with the ratio of 1:5 after restriction enzyme digestion. 1 U of T4 DNA ligase (Promega) was used in every 10 µl of the total volume of reaction. The mixture was incubated at 4°C overnight.

2.2.1.3 Transformation

TOP 10 *E.coli* strain was used for propagation of all the plasmid DNA. An aliquot (90 µl) of Top 10 cells and 1 µl of DNA plasmid were used for each reaction. Plasmid DNA was mixed with bacteria and incubated on ice for 30 min. Thereafter they were subjected to heat shock at 42°C for 45 sec before being replaced on ice for 2 min. Subsequently, 800 µl of LB medium was added to the cell mixture and incubated in an orbital shaker incubator (225 rpm) at 37°C for 1 h. Finally, the bacteria were plated onto LB agar containing appropriate antibiotics and incubated at 37°C for 16 h.

2.2.1.4 Small-scale isolation of plasmid DNA

A single colony was picked from the LB agar plate from the transformation procedure and inoculated into a 3.5 ml LB liquid culture containing the same antibiotic and grown for 16 h in an orbital shaker incubator (225 rpm) at 37°C.

Plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen) as recommended by the manufacturer.

2.2.1.5 Large-scale isolation of plasmid DNA

A single colony was picked from the LB agar plate from the transformation procedure, inoculated into a 3.5 ml LB liquid culture that also contained the appropriate antibiotic, and grown in an orbital shaker incubator (225 rpm) for 8 h at 37°C. A 0.4 ml aliquot of this culture was then added to 200 ml of fresh LB with antibiotic and grown for 16 hs at 37°C. Plasmid DNA was isolated using QIAfilter Maxiprep Kit (Qiagen) according to manufacturer's instructions.

2.2.1.6 Quantification of DNA

Plasmid DNA was diluted 1:200 in sterile ddH_2O and quantified by optical diffraction (OD) at 260 nm on an Ultrospec 2100 pro UV/Vis spectrometer (Amersham Biosciences). The OD ratio at 260 nm/280 nm gave an indication of DNA purity, where a ratio of 1.8-2.1 represented the highly purified DNA.

2.2.1.7 Gel extraction of DNA

Isolation of PCR products which represented < 10 μg DNA was achieved following an initial agarose electrophoresis step, as described in 2.2.1.1. The DNA fragments in agarose-gel were visualized by UV 320 nm. Gel slices containing the DNA fragment

of interest were cut out and extracted using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction.

2.2.2 Reporter gene constructs

2.2.2.1 Introduction of point mutations in the promoter region of mouse *Nqo1*

The WT -1016/Nqo1-Luc reporter construct, which contains 1016 bp of the upstream region of the mouse *Nqo1* gene, has been described previously (Nioi et al., 2003). Transversion point mutations were introduced to the ARE consensus sequence in which -435 5'-TGAG-3' -432 was mutated to -435 5'-GTCT-3' -434 to generate ARE_{mut}-1016/Nqo1-Luc, or XRE consensus sequence in which -385 5'-GCGTC-3' -380 was mutated to -385 5'-TATGT-3'-380 to generate XRE_{mut}-1016/Nqo1-Luc. To generate ARE_{mut} -1016/Nqo1-Luc, the pair of primers used are ARE 5': 5'-CTTTCAGTCTAGAGTCACAGGTCTTCGGCAAAATTTGAGCCC-3' and ARE 3': 5'-GGGCTCAAATTTTGCCGAAGACCTGTGACTCTAGACTGAAAG-3'. The pair of primers used for generating XRE_{mut}-1016/Nqo1-Luc, were XRE 5' 5'- CCC CACCCTTCCCCTATATGTCAAAGGTGACTTCCCACGGC-3' and XRE 3' 5'-GCCGTGGGAAGTCACCTTTGACATATAGGGGAAGGGTGGGG-3'. The simultaneous mutation of both ARE and XRE was generated by using ARE_{mut}-1016/Nqo1-Luc as the template and XRE 3' and XRE 5' as the primer pair. Point mutagenesis was performed using polymerase chain reaction and Hot start Pfu

turbo DNA polymerase (Stratagene, Cheshire, UK) to amplify the target sequence. Three steps PCR were used to amplify the DNA. Firstly, template DNA was denatured at 95°C for 2 min. Secondly, the DNA template was denatured at 95°C for 1 min, before the primers were annealed at 55°C for 1 min, and then extended at 68°C for 12 min; these events were repeated for 25 cycles. Lastly, the DNA was extended at 68°C for 15 min for 1 cycle. The components of the reaction mixture are listed in table 2.3. After confirming the accuracy of the mutagenesis and whole sequence by DNA sequencing the PCR fragments were digested

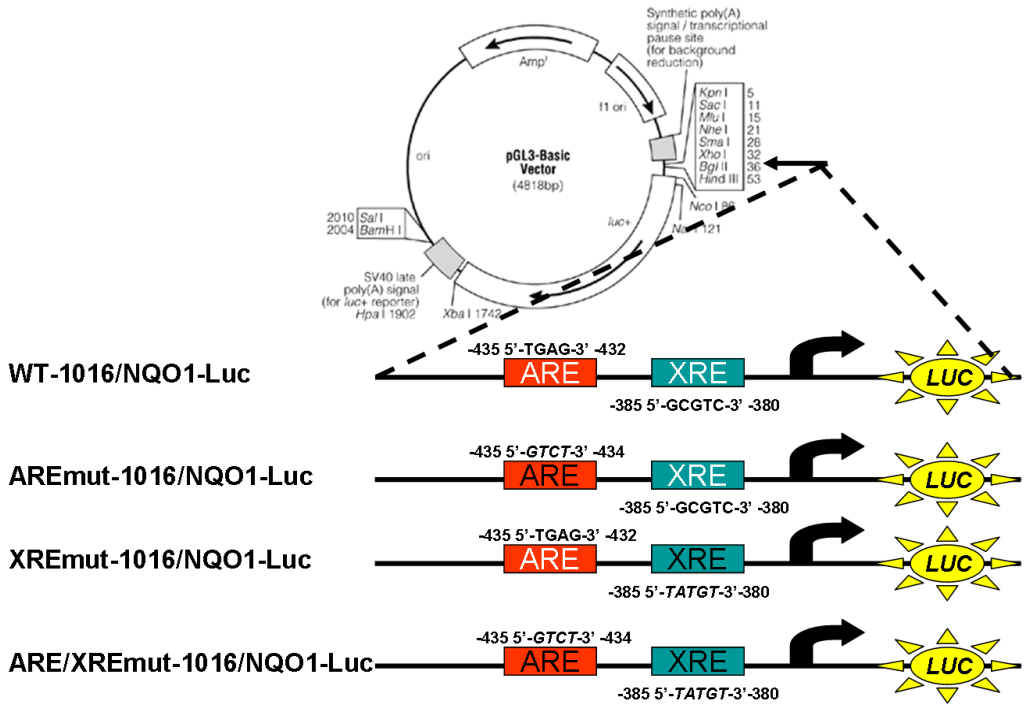


Figure 2.1 Generation of construct containing transversion mutations.

Strategy for generating construct containing transversion mutations of ARE, XRE or both of these element is described in material and methods. White letters indicate the original element sequence, while black letters and the sequence in italic either above (ARE) or under the box indicated the mutated sequence. The map of pGL-3-Basic vector into which the promoter sequence ligated was shown here as well.

2.2.2.2 Deletion of the ARE and XRE sequences in the promoter region of mouse NQO1

Deletion mutations were introduced using $P_{-1016/Nqo1}$-Luc reporter construct as the template to remove either the ARE (-435 5'-TGAGTCGGC-3' -427) or the XRE (-387 5'-TAGCGTG-3'-380) in the 5'-upstream region of *Nqo1* to generate the constructs $\Delta ARE_{-1016/Nqo1}$-Luc and $\Delta XRE_{-1016/Nqo1}$-Luc respectively. Deletion mutagenesis was performed using the polymerase chain reaction and Hot start pfu turbo DNA polymerase (Stratagene, Cheshire, UK) to amplify the target sequence. For $\Delta ARE_{-1016/Nqo1}$-Luc, the pair of primer used are 5'-CAGTCTAGAGTCACAGAAAATTTGAGCCCATCC-3' (sense) and 5'-GGATGGGCTCAAATTTTCTGTGACTCTAGACTG (antisense). To generate ΔXRE-1016/Nqo1-Luc, the primer pair used were 5'-GCCCCACCCTTCCCCCAAAGGTGACTTCCCACG-3' (sense) and 5'-CGTGGGAAGTCACCTTTGGGGGAAGGGTGGGGC-3' (antisense). Deletion of XRE was introduced within $\Delta ARE_{-1016/Nqo1}$-Luc to make a double deletion construct $\Delta ARE/XRE_{-1016/Nqo1}$-Luc by using the same primer pair that was employed to generate $\Delta XRE_{-1016/Nqo1}$-Luc. Three steps PCR were used to amplify the DNA as describe in 2.2.1.1. The component of the reaction mixture is listed in table 2.3.

Components	Final concentration
DNA template	2 ng/µl
Primer	3 ng/µl
10mM dNTP mixture	0.3 mM
Pfu reaction buffer (5×)	1×
Hot start pfu turbo DNA polymerase (2.5 U/µl)	0.05 U/µl

Table 2.3 Components and their final concentration the of PCR mix for point and deletion mutagenesis of the ARE and XRE sequences.

2.2.2.3 XRE luciferase reporter plasmids

The XRE-luciferase reporter plasmids were generated using the pGL3-promoter vector (Promega) containing an SV40 promoter upstream of the firefly luciferase gene. Either two or four copies of the XRE (5'-GTGCG-3') present in the mouse Nqo1 promoter were inserted into the vector in a head-to-tail orientation, through *Nhe*I and *Xho*I restriction sites upstream of the promoter-luc transcriptional region. A linker with the sequence of 5'-CCC-3' and 5'-GGG-3' was placed between individual XRE enhancers. Plasmids generated were called pGL_2×XRE-Luc or pGL_4×XRE-Luc. Primer pairs used for generating pGL_2×XRE-Luc or pGL_4×XRE-Luc were XRE2×_For (5'-CTAGCGCGTGGGGGCGTGC-3') and XRE2×_Rev (5'-TCGAGCACGCCCCACGCG-3') or XRE4×_For (5'-CTAGCGCGTGGGGGCGTGGGGGCGTGGGGGCGT) and XRE4×_Rev (5'-TCGAGCACGCCCCCACGCCCCCACGCCCCCACGCG-3') respectively. All primers have a phosphate modification at the 5' end and were diluted in ddH_2O to 100 pmol/l. An alquot of 20 µl of each primer of the primer pair was mixed with 5 µl

of 10 × annealing buffer (100mM Tris, pH7.5, 1 M NaCl and 10 mM EDTA) in a total volume of 50 µl and incubated at 100°C for 5 mins followed by 1 h incubation at 20°C. Annealed primers and the pGL3-promoter vector were digested by *Xho*I and *Nhe*I restriction enzymes before being ligated .

2.2.2.4 Expression constructs for AhR, Nrf2, Keap1 and Ubiquitin

Expression constructs for mouse Nrf2-V5, mKeap1 and his-ubiquitin were generously provided by Dr. Michael McMahon.

Expression construct for mouse AhR was generated as stated below. A cDNA clone containing the entire coding region of mouse AhR (100016223) was obtained from Geneservice (Cambridge, UK) and was located in the pENTR223.1 vector between two Sfi I sites. The plasmid was first purified from bacteria clone using QIAprep Spin Miniprep kit (Qiagen, Sussex, UK). The accuracy of the inserted cDNA sequence encoding the whole region of Mouse AhR was verified by sequencing using M13F primer. The cDNA encoding mouse AhR was amplified with hot start turbo DNA polymerase using the two oligonucleotides 5'-CCCAAGCTTGGCACCATGAGCGGCGCCAACATC-3' (sense) and 5'-CCGCTCGAGACTCTGCACCTTGCTTAGGAATGC-3' (antisense) to introduce a 5' *HindIII*and a 3' *xhoI* restriction sites. A two-step PCR amplification was carried out: in the first step, the DNA template was denatured at 95°C for 1 min for 1 cycle; in the second step, the DNA template was denatured at 94°C for 1 min, annealed to primers at 68°C for 30 sec and extended at 68°C for 3 min (this was repeated for 35

cycles). The final PCR product was digested using restriction enzymes *HindIII* and *XhoI* (Promega) and ligated into digested pcDNA3.1/V5 his A that had been treated with the two restriction enzymes.

2.2.3 DNA sequencing

The fidelity of the cDNA products and all constructs made in this study were sequence-verified by the Human Genome Group, Department of Molecular and Cellular Pathology, Medical School University of Dundee, Scotland, UK.

2.2.4 Transient transfection experiments

Transfection experiments were usually conducted in RL-34 cells or MEFs. Typically, 6×10^5 cells/well were seeded in 6-well plates and allowed to grow overnight to reach the confluence of 70%. Cells were then transfected with 2 μg total amount of DNA using lipofectamine^{TM} 2000 as the transfection reagent and β-gal construct was used as an internal control. The ratio of β-gal construct to target construct was 1:15. For over-expressing experiment, pcDNA3.1/A was added to the transfection mixture to ensure equal amount of total DNA of every sample. The transfection procedure was as follows: 2 μg of plasmid DNA was diluted in 100 μl Opti-MEM and 4 μl (RL-34 cells) or 6 μl (MEF cells) lipofectamine^{TM} 2000 reagent was diluted in 100 μl Opti-MEM. The mixture was incubated for 5 min at RT. Thereafter diluted DNA was added to diluted transfection reagent and incubated for 15 min at RT. Meanwhile,

cells were washed with PBS twice and media was replaced with 800 µl Opti-MEM. After incubation, 200 µl DNA-lipofectamine complex was added to cells in each well and left for 6 h before medium was changed back to growth medium. Cells were then recovered in growth medium overnight and used for later experiment.

2.2.5 Luciferase reporter gene assays and statistical analysis

For AREc32 cells, after treatment, luciferase activity was measured using Steady-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions. For RL-34 and MEF cells, after transfection and treatment, luciferase activity was measured using Luciferase Reporter Assay System (Promega). Cells were washed once with RT PBS and lysed in 1 × passive lysis buffer. Lysate was then centrifuged using a bench top centrifuge 13000 rpm for 1 min to remove cell debris. 20 µl supernatant was used for measuring luciferase activity according to the manufacturer's protocol.

2.2.6 TaqMan quantitative real-time PCR

2.2.6.1 Isolation of RNA from cultured cells

The cell monolayer was washed twice with ice-cold PBS and scraped into lysis buffer RLT supplied with the RNeasy Mini Kit (Qiagen) (350 µl/60mm dish) containing 1% (v/v) β-mercaptoethanol. Isolation of RNA was then achieved using

the RNeasy Mini Kit, including the on-column DNA digestion step.

2.2.6.2 Isolation of RNA from animal tissues

Tissues were pulverized to a fine powder using a pestle and mortar under liquid nitrogen. Samples (10 – 30 mg) were placed in an Eppendorf microfuge tube, which was pre-chilled on dry-ice, and homogenised in 600 µl ice-cold RLT containing 1% (v/v) β-mercaptoethanol using an Ultra-Turrax T8 rotor-stator homogeniser (IKA-Werke, 60 sec at 90% power). Homogenised samples were centrifuged at 16,000 *g* at 4°C for 10 min to remove debris and the supernatants were loaded onto RNeasy spin columns for isolation of RNA using the RNeasy Mini Kit (Qiagen), including the on-column DNA digestion step.

2.2.6.3 Quantification of RNA

RNA was diluted 1:200 in sterile ddH_2O and quantified by OD_{260} on an Ultrospec 2100 pro UV/Vis spectrometer (Amersham Biosciences). As was the case for DNA, the OD ratio at 260 nm/280 nm gave an indication of RNA purity, where 1.8 – 2.0 is the ideal range. Quality of RNA was assessed by agarose-gel electrophoresis. Two clear visible distinct bands corresponding to 28S and 18S rRNA would indicate that the RNA was of good quality.

2.2.6.4 Reverse transcription of mRNA to cDNA

RNA was reverse-transcribed to cDNA using the Omniscript Reverse Transcription Kit (Qiagen). At this stage, concentration of RNA of each sample was equalized and 1.5 µg of RNA was used for each reaction; oligo (dT) 15 primer (Promega) was diluted in ddH_2O to the final concentration of 10 µM. The component for each reaction was listed in Table 2.4. The reaction was preceded for 1 h at 37°C, whereupon cDNA was diluted in ddH_2O to a final concentration of 10 ng/µl.

Components	Volume	Final concentration
10 × Buffer RT	2 µl	1 ×
dNTP Mix (5 mM each)	2 µl	0.5 mM each
Oligo (dT) 15 primer (10 µM)	2 µl	1 µM
RNase inhibitor (20 u/µl)	0.5µl	0.5 U/µl
Omniscript reverse transcriptase (4 U/µl)	1 µl	0.2 U/µl
Rnase-free water	Variable	
Template RNA	Variable	1.5 ug (per 20 µl reaction)
Total volume	20 µl	

Table 2.4 Components and their volume and final concentration for reverse-transcription reaction.

2.2.6.5 Quantitative Real-Time PCR (TaqMan®)

TaqMan® Gene Expression Assays (table 2.4) was used to quantify mRNA for NQO1, Nrf2, AKR1C1, CYP1A, AhR. All the assays were from Applied Biosystems including as assay for actin mRNA, employed as an internal control. All the assays were labelled with a 5' fluorescent reporter dye (6-carboxyfluorescein (6-FAM)) and a 3' quenching dye (6-carboxytetramethylrhodamine (6-TAMRA)).

Quantitative Real-Time PCR (qRT-PCR) was performed on the Prism Model 7700

Sequence Detector (Perkin Elmer/Applied Biosystems). Samples were analyzed in triplicate. Each reaction mixture had a final volume of 15 µl and contained 0.75 µl TaqMan® Gene Expression Assay, 4.5 µl master mix, and 20 ng of cDNA.

The TaqMan® reaction conditions comprised an initial cycle of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec then 60°C for 1 min. Fluorescence at 518 nm was measured, with excitation at 494 nm. Threshold cycle (C_T) values, where the PCR product became detectable above the background signal, were analyzed using Sequence Detector v1.7 software (Perkin Elmer Biosystems). Fold-induction values were normalized against a control treatment and were calculated relative to that of actin calibrator in Excel (Microsoft) using the comparative C_T method.

Gene symbol	Gene name	Issay ID	Species
NQO1	NAD(P)H dehydrogenase, quinone 1	Mm00500821_m1	M.musculus
NFE2L2	Nuclear factor, erthroid derived 2, like 2	Mm00477786_m1	
CYP1A1	Cytochrome P450, family 1, subfamily a, polypeptide 1	Mm00487218_m1	
AHR	Aryl-hydrocarbon receptor	Mm01291777_m1	
NQO1	NAD(P)H dehydrogenase, quinone 1	Rn01640845_m1	R.norvegicus
NFE2L2	Nuclear factor, erthroid derived 2, like 2	Rn00582415_m1	
CYP1A1	Cytochrome P450, family 1, subfamily a, polypeptide 1	Rn00487218_m1	
AHR	Aryl-hydrocarbon receptor	Rn01645056_m1	
AKR1C1	Aldo-keto reductase family 1, Member C1	Hs00413886_m1	H.sapiens

Table 2.5 Assay ID of the gene expression assays.

2.3 Cell biology methods

2.3.1 Cell lines and culture media

All cell lines were grown at 37°C in 5% CO_2 and 90% relative humidity. AREc32 cells were generously provided by Dr. XiuJun Wang (Biomedical Research Institute, Medical school, University of Dundee, Ninewells Hospital, Dundee). These cells were derived from the MCF-7 cell line following stable transfection with a luciferase reporter construct driven by 8 copies of the ARE found in mouse Gsta1 that was ligated into the pGL3-promoter vector (Wang *et al.*, 2006). The AREc32 cells were maintained in DMEM (1 mg/ml glucose) supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin and 0.8 mg/ml G418. Cells were passaged upon reaching ~70% confluence. The cell monolayer was washed with PBS and incubated with 0.25% (w/v) trypsin in PBS for 5 – 10 mins. Cells were resuspended in growth medium and pelleted by centrifugation, resuspended in growth media and split 1:4 or 1:3.

Rat Liver RL-34 cells, Monkey kidney COS1 cells, human endometrial adenocarcinoma HEC-116 cells, human epithelial colorectal adenocarcinoma cells and human colon adenocarcinoma HT29 cells lines were maintained in DMEM (4.5 mg/ml glucose) supplemented with 10% FBS and 1% (V/V) penicillin/streptomycin. Murine hepatoma hepa-1c1c7 cells were maintained in MEM alpha medium without nucleoside supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin. The cell monolayer was washed with PBS and incubated with 0.25% (w/v) trypsin in PBS for 5 – 10 min. Cells were resuspended in growth medium and pelleted by

centrifugation, resuspended in growth media and split 1:6 to 1:3.

MEF cells were maintained in IMDM (17.7g powdered IMDM (Invitrogen), 40.4 ml 7.5% $NaHCO_3$, made up to 1 L in sterile dH_2O) supplemented with 10% and 0.001% (w/v) EGF (Invitrogen) and 5% (v/v) 100 × ITS (Gibco). MEF cells were maintained in flask or dishes coated with 0.1% gelatine (Sigma). Cells were passaged every two or three days. The cell monolayer was washed with PBS and incubated with 0.05% (w/v) trypsin with 0.2 mM EDTA in PBS for 5 min RT. Cells were resuspended in growth medium, pelleted by centrifugation (1500 rpm, 3 min), resuspended in growth media and split 1:2.

2.3.2 Treatment of cells with chemicals

1000 × stock solutions of all compounds were prepared in dimethyl sulphoxide (DMSO). For treatment, stocks were diluted 1:1000 into complete media, giving a final concentration of DMSO vehicle in the media of 0.1% (v/v).

Once AREc32 cells reached to the confluence of 70%, growth medium was replaced with growth medium without serum containing appropriate chemicals and left for 24 hours.

For RL34, HT-29, HCT116, Caco2 and MEF cells, growth medium was replaced with fresh growth medium with appropriate chemicals. For luciferase assay, cells were treated for 24 hours. For Western blot analysis of NQO1 and AKR1C1, cells

were treated with chemicals for 24 hours while for the analysis of Nrf2, 2 hours treatment was carried out. For Taq-man experiment, cells were treated for 12 hours.

2.3.3 Immunocytochemistry

RL-34 cells were grown as a monolayer on coverslips that were placed in 60mm dish and allowed to reach 40% confluence. Medium was then replaced with growth medium containing appropriate chemicals for either 30 min or an hour. After chemical treatment, cells were firstly washed with cold 1× PBS (0.01 M phosphate buffer (pH6.8), 0.14 M NaCl) twice and then fixed with 1 ml of 4% (w/v) paraformaldehyde for 3 min. After the incubation, cells were washed three times in 1 × PBS each for 5 minutes and subsequently permeabilized with 1 ml of 0.2% Triton X-100 for 20 min at RT. Cells were then washed 3 times as described before and incubated with 1 ml of blocking buffer (3% BSA in 1 × PBS) for 1 hour at 4°C. In succession, the blocking buffer was replaced with primary antibody (1:200 dilution for Nrf2 antibody and 1:50 dilution for AhR antibody), and left to react for overnight at 4°C. The coverslips were then washed as before and subsequently incubated with 1 ml blocking buffer containing the fluorescent –labelled secondary antibody. Alexa-Fluor 48 (FITC, fluorescein isothiocyante)-Goat anti-rabbit IgG (Invitrogen) was used as the secondary antibody for Nrf2. Alexa-Fluor 594 (Rhodamine) donkey anti-sheep IgG was used as the secondary antibody for AhR.

The cellular DNA was stained by incubating the slides in a solution of

4’-6-diamidino-2-phenylindole (DAPI, 10 μg/ml) for 10 min at RT. The coverslips were washed 3 times as before and mounted on a glass slide using 10% (w/v) Mowiol medium and were allowed to dry at 4°C. The cell-loaded slides were examined under a confocal microscope.

2.3.4 Confocal microscopy

The cells were labelled with appropriate primary and secondary antibodies for immunocytochemistry. FITC (excitation/emission = 495/519 nm) or Rhodamin (excitation/emission = 594/610 nm)-labelled secondary antibody was used to locate the endogenous proteins. Confocal images were obtained using a LSM 510 laser scanning microscope system (Carl Zeiss, Germany). The DAPI image represents nuclear DNA staining, DIC indicated images from normal light microscopy, and the merge signal represents the results obtained when the three images were superimposed.

2.3.5 Subcellular fractionation

2.3.5.1 RL-34 cells

RL-34 cells were seeded in two 100 mm dishes for each subcellular fraction sample to reach the confluence of 100%. Afterwards, cells were treated with appropriate chemicals for 2 h and preceded for subcellular fractionation. The procedure is described below.

Cells were washed once with ice cold PBS, scraped in serum free DMEM and centrifuged at 600 × *g*, 4°C for 5 min. Medium was then removed and the cell pellet was homogenized in 4 pellet-volumes of 1× Isotonic buffer (10 mM Hepes, pH7.8, containing 250 mM, 1 mM EGTA, 1mM EDTA and 25 mM KCL) supplemented with 1% (w/v) complete protease inhibitor cocktail (Roche Applied Science). The resuspended cells were gently homogenized by repetitively passing the mixture through a 23-gauge needle with 20 strokes. Afterwards, the homogenate was centrifuged at 1300 × *g* at 4°C for 15 min both pellet and supernatant were kept. The cell pellet was resuspended in 500 µl STM buffer (250 mM sucrose, 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$ and 10 mM iodoactamide) by gently pippeting and layer onto 200µl of a sucrose cushion (40% (w/v) sucrose, 10 mM HEPES (pH7.5), 10 mM KCL, 1.5 mM $MgCl_2$ and 1 mM dithiothreitol). Resuspended cells were subsequently spanned at 1000 × g and 4°C for 15 min and the pellet acquired was the purified nuclei (N) which was subjected to be lysed in 100 µl RIPA buffer supplemented with 1% (w/v) complete protease inhibitor cocktail. Meanwhile, supernatant obtained from the 1300 × *g* centrifugation was centrifuged again at 17000 × *g* 4°C for 30 min to remove cell debris. The supernatant was then centrifuge at 100, 000 × *g* 4°C for 1 hour and the pellet is kept as membrane fraction (M) which was homogenized in 100 µl RIPA buffer while the protein in the supernatant fraction was and kept as the cytosolic fraction (C) and concentrated by acetone precipitation,

2.3.5.2 MEF cells

The procedure of cell fractionation for MEF cells is similar with that for RL-34 cells with some modification and the steps are shown below. In addition, only purified nuclei and 3000 × *g* fraction containing membrane and cytosol are separated.

Cells was washed once with ice-cold PBS, scraped in serum free IMDM and centrifuged at 1500 × *g*, 4°C for 5 min. Medium was then removed and cell pellet was resuspended in 4 pellet-volumes of 1× hypotonic buffer (10 mM Hepes, pH7.8, containing 250 mM, 1 mM EGTA, 1mM EDTA and 25 mM KCL) supplemented with 1% (w/v) complete protease inhibitor cocktail (Roche Applied Science). Resuspended cells were kept on ice for 30 min and subsequently gently homogenized by repetitively passing the mixture through a 25-gauge needle with 25 strokes. The homogenate was then centrifuged at 3000 × *g* at 4°C for 10 min; the supernatant is kept as fraction (S) which contains cytosol and membranes. Protein from the supernatant was concentrated by acetone precipitation as described previously. Meanwhile, the cell pellet was resuspended in 500 µl STM buffer by gently pippeting and layer onto 200µl of a sucrose cushion and subsequently spanned at 3000 × *g* and 4°C for 10 min. The pellet was the purified nuclei (N) and was lysed in 100 µl RIPA buffer supplemented with 1% (w/v) complete protease inhibitor cocktail.

2.4 Biochemical methods

2.4.1 Cytotoxicity assay

The cytotoxicity of phytochemicals to cells was examined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as modified by (Hansen *et al.*, 1989). This technique exploits the formation of a water-insoluble purple formazan product generated from water-soluble yellow MTT by functional mitochondria as a means of spectrophotometrically measuring viability following dissolution of the cell monolayer in organic solvent. RL-34 or Hepa1c1c7 cells were seeded in 96-well plates at the density of 8×10^3 cells per well in 100 µl growth medium, giving ~80% confluence after 24 h incubation. Media was then removed carefully by gentle inversion and replaced with 100 µl of treatment media containing different concentration of the phytochemical of interest in growth media. After 20 h incubation at 37°C, 25 µl MTT (5 mg/ml in sterile PBS) was added to each well, giving final concentration of MTT of 1 mg/ml before the mixture was incubated at 37°C for 90 min. A volume of 100 µl lysing buffer (Dissolve 20% (w/v) SDS into 50% (v/v) N, N-dimethylformamide DMF in ddH_2O and adjust pH to 4.7) was added to each well subsequently and incubated at 37°C for 60-90 min to allow all the formazon dissolve completely. Plates were then read at 570 nm on a Benchmark microplate reader (Bio-Rad) and lysing buffer was used as blank. Data were analyzed using Excel software, wherein absorbance was converted to arbitrary units of survival.

2.4.2 Measurement of NQO1 enzyme activity assay

RL-34, MEF Nrf2$^{+/+}$ and MEF Nrf2$^{-/-}$ cells were seeded in 96-well plates with a starting density of 1.7×10^4 or 2×10^4 cells per well for RL-34 or MEF cells respectively. Thereafter, the cells were incubated for ~24 hs to reach the confluence of ~80%. The cell culture media was removed by inverting the plates and was replaced with growth media containing appropriate chemicals before being left for another 24 h at 37°C, 5% CO_2. The media was then removed and the plates were washed with PBS twice, after which the cells were lysed in 75 µl/well digitonin (a suspension of 0.8 g/L digitonin in PBS containing 2 mM EDTA, pH7.8) at RT for 30 min. During the last 10 min of lysis, the plates were transferred to the orbital shaker at 150 rpm. By the completion of lysis, 20 µl of the cell lysates were transferred to a fresh 96-well plate and protein concentration was measured by BCA assay. The remaining cell lysates were used for NQO1 enzyme activity assay according to "Prochaska" microtiter plate bioassay (Fahey *et al.*, 2004). The procedure of this assay is described as follows. A volume of 200 µl MTT buffer (0.025 M Tris-Cl, pH 7.4, 0.5% BSA (w/v), 0.01% Tween 20, 5 µM FAD, 1mM G-6-P, 30 µM NADP, 20 U/ml G-6-P-D, 0.3 mg/ml MTT and 4.3 µg/ml Menadione (dissolve menadione in AcN at 4.3 mg/ml)) was added to each well and plates were then incubated at RT for 5 min followed by addition 50 µl dicumarol (5 % DMSO, 5 mM KPO_4 and 0.3 mM dicumarol). The plate was then immediately read at absorbance of 610 nm using SpectraMax (Molecular devices, 1311 Orleans Drive, Sunnywale, CA 94089-1136, USA). A every 30 sec Kinetic reading lasting for 5 min was performed at first and 5

min endpoint reading was chosen and carried out for the analysis. Absorbance obtained was normalized by protein concentrations and the change of NQO1 activity was calculated.

2.4.3 Determination of protein concentration

2.4.3.1 Determination of protein concentration by Bradford assay

Protein concentration was determined according to the method of Bradford assay (Bradford, 1976). Samples were compared to a standard curve of bovine serum albumin (BSA) dissolved in water at concentration of 0, 0.0625, 0.125, 0.25 and 0.5 mg/ml. Bradford reagent (Bio-Rad) was diluted 1:5 with dd H_2O. Samples and Bradford reagent were then loaded to RX DAYTONA machine (Randox Laboratories Ltd., 55 Diamond Road, Crumlin, Co. Antrim, UK) which measured the absorbance at 595 nm for calculation of protein concentration within samples.

2.4.3.2 Determination of protein concentration by Bio-Rad *DC* protein assay

For each assay, mix 2µl reagent S with 100 µl reagent A which formed the solution A'. Add 4 µl of lysate or standard which is the same as describe in section 2.4.3.3 into each plastic cuvette. Subsequently add 100 µl of A' followed by 800 µl of reagent B and incubate at RT for 15 min. Absorbance was read at OD750nm by an Ultrospec 2100 pro UV/Vis spectrometer. Protein concentration was calculated according to the standard curve.

2.4.4 Western blotting

2.4.4.1 Preparation of protein samples from cells

Cells were seeded in 60 mm dishes and treated with xenobiotic as described in 2.3.2. At the end of each experiment, the cells were washed twice in ice-cold PBS and scraped into 400 μl ice-cold Radio-immune Precipitation Buffer (RIPA) (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% (v/v) NP40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS), supplemented with complete EDTA-free protease inhibitor cocktail (1 tablet/10 ml). The cell lysates were incubated on ice for 15 min followed by centrifugation at 13,000 rpm at 4°C for 20 min to remove insoluble cell debris. The protein concentration of the supernatant was measured by Bradford assay. 1:10-1:20 dilution was made to supernatant for protein concentration analysis.

2.4.4.2 Protein preparation from animal tissues

10-30 mg tissue powders prepared in section 2.2.6.2was measured out and put into an Eppendorf microfuge tube pre-chilled on dry ice. Add 1 ml 1 × SDS buffer (0.125 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol) to every 10 mg of sample. Samples were homogenized on ice for 1 min using an Ultra-Turrax T8 rotor stator homogenizer (IKA-Werke, 90% power). Homogenized samples were incubated at 70°C with shaking at 1400 Hertz for 10 min. Any undissolved tissue residual was sedimented by centrifugation at 16,000 *g* at RT for 10 min to remove insoluble debris.

Protein concentration of the supernatant was analyzed by Bio-Rad *DC* protein assay. After protein concentration analysis, 0.001% bromophenol blue was added to the sample.

2.4.4.3 Polyacrylamide gel electrophoresis (PAGE)

Protein samples were resolved using the discontinuous electrophoresis method of Laemmli (Laemmli, 1970). Cell lysates were adjusted to final concentration of 1 mg/ml in SDS-PAGE sample buffer (5× solution: 67mM Tris-HCl, pH 6.8, 2.2% (w/v) SDS,27% (v/v) glycerol, 0.72M β-mercaptoethanol , 0.1% (w/v) bromophenol blue) and denatured by heating for 4 min at 95°C.

Polyacrylamide gels (0.75 cm thick) were cast in the Mini-Protein II cell (Bio-Rad). Separating gel (8-12% (w/v) acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) TEMED and 0.1% (w/v) ammonium persulphate) were poured and overlaid with ethanol until polymerisation was complete (10 min). Ethanol was then removed and stacking gel (4% (w/v) acrylamide, 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) TEMED, 0.1% (w/v) ammonium sulphate) pored directly on top of the separating gel. Gel combs were inserted into stacking gel to create sample wells, and polymerisation was allowed to proceed (15-30 min). Samples were loaded into wells for electrophoresis in running buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS) at 200V for 45-60 minutes to achieve good separations of protein.

2.4.4.4 Transfer of proteins to Immobilon-P™ membrane

Proteins from SDS-PAGE acrylamide gels was transferred to Immobilon-P™ membrane (Millipore) using the Mini Transblot System (Bio-Rad) and carried out in transfer buffer (25 mM Tris, 200 mM glycine, 20% (v/v) methanol), which was kept cool by an icepack, at 100V for 1 h. For protein with large molecular mass (i.e. ubiquitinated Nrf2 protein), 0.1% SDS (w/v) was included in the transfer buffer.

2.4.4.5 Immunoblotting with primary and secondary antibodies

All incubations were performed in petri-dish placed on an orbital shaker at 60 rpm. Immobilon-PTM membranes were blocked in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween-20®) with 10% (w/v) powdered fat-free milk (Marvel) overnight at 4°C. Primary antibody, appropriately diluted in 10% TBS-T/milk was then added, again for incubation either overnight at 4°C or for 1 h at room temperature. The membrane was then washed first in 0.25% TBS-T once for 5 min and then in 0.1% TBS-T 3 x 5 min at RT before addition of the appropriate horseradish peroxidise (HRP)-conjugated secondary antibody in TBST/milk for incubation for 1 h at room temperature, followed by a second series of washes as described before.

2.4.4.6 Visualization of antibody complex by enhanced chemiluminescence

Antibody-protein complexes on Immobilon-P™ membranes were visualized by

enhanced chemiluminescence (ECL) and autoradiography. Following the final series of washes, membranes were incubated for 30 s in equal volumes of ECL solution 1 (100 mM Tris, pH 8.5, 2.5 mM luminol, 0.4 mM *p*-coumaric acid) and ECL solution 2 (100 mM Tris, pH 8.5, 0.02% (w/v) H_2O_2), then directly exposed to autoradiographic film (Super RX, Fuji). Quantitation of the chemiluminescent signal intensity required use of a commercial ECL solution (Immobilon Western Chemiluminescent HRP Substrate (Millipore)), followed by signal detection in an LAS-3000 mini Imager (Fujifilm). Data were analyzed using Quantity One Software (Bio-Rad). Intensity of the band for target protein was normalized to that of actin or GAPDH.

2.4.4.7 Re-probing western blots

Some antibody-blotted membranes were washed for 30 min in 0.1% TBS-T followed by incubation with a stripping buffer (7 M guanidine hydrochloride, 50 mM glycine, 0.05 mM EDTA, 0.1 M KCl and 20 mM 2-mercaptoethanol at pH 10.8) for 8-10 min. Membranes were then washed under running tap water and 0.1% TBS-T for 20 min at RT. The membranes were then re-probed with additional primary antibody. The subsequent steps of the immunoblotting procedure were carried out as described before.

2.4.5 Protein precipitation by acetone

In order to concentrate proteins in cell lysates, 5 × volume of acetone was added to the sample. The mixture was kept on ice for 5-15 min before being spun at 3000 × g for 5 min. The supernatant was removed and the pellet was dissolved in an appropriate volume of RIPA buffer by sonication (2 × 10 sec pulse at 8 W).

2.4.6 Determination of the half life of Nrf2 protein

RL-34 cells were treated with quercetin, kaempferol or DMSO for 2 hours prior to treatment with cycloheximide (final concentration 1μmol/l) for between 0 and 60 min. Whole cell lysates were prepared and the amount of Nrf2 protein was examined by Western blotting. Densitometry analysis was then carried out using Quantity One Software (Bio-Rad) to calculate band intensities. The relative amount of Nrf2 was plotted on a semi-log plot with the amount of Nrf2 protein obtained before the addition of CHX being set at 1.0.

2.4.7 Protein co-immunoprecipitation experiment

RL-34 cells were seeded in 100 mm dish and left for ~ 24 hours to reach 100% confluence before they were treated with appropriate phytochemicals at various time points prior to being harvested at the same time. Cells were then washed twice with ice-cold PBS and scraped in 400 μl lysis buffer (50 mM Tris, pH 8.0, 2 mM EDTA, pH 8.0, 1 mM $NaVO_4$, 50 mM NaF, 2.5% glycerolphosphate (v/v), 10% Triton

X-100 and 0.1 mM PMSF). Once in lysis buffer, cells were incubated on ice for 20 min, after which, the lysates were centrifuged at 14,000 rpm at 4°C for 20 min. Protein concentration was measured using the supernatant. An aliquot of 40 µl of the supernatant was added into another eppendorf containing 40 µl H_2O and 20 µl 5 × reducing Laemmli sample buffer followed by incubating at 95°C for 4 min and this was the input. For the rest of the supernatant, 1 mg of protein from each sample was mixed with 2 µg immunoprecipitation antibody and was left tumbling en-over-end at 4°C overnight. After the overnight incubation, the mixture was added to 50 µl of protein agrose A/G and left for tumbling for 2h before being washed three times, 5 minutes each. Finally in lysis buffer 50 µl 2 × reducing Laemmli buffer and boil the sample as before.

2.4.8 In-vitro ubiquitin assay

COS-1 cells were seeded in 60 mm dishes at 1×10^6 cells/dish and left to recover for 20-24 hours. Cells were then transfected as described before with appropriate combinations of plasmids including pcDNA3.1/V5mNrf2, mKeap1 and pHis-Ub. The empty pcDNA3.1 plasmid was used to equalize the total amount of DNA transfected to cells in each dish. On the third day, cells were treated with various phytochemicals for 2 h followed by ubiquitination assay. The procedure of the assay is stated as below.

After removal of medium cells were washed with one volume of ice-cold PBS and scraped into 0.4 ml of ice-cold PBS. An aliquot of the suspension (80 µl) was transferred to a separate eppendorf and the subjected for centrifugation at 500 x *g*, 4^0C for 1 to pellet the cells. The cell pellet was subsequently lysed in 200 µl of 2x reducing Laemmli sample buffer and sonicated (2 x 10 sec pulses at 8 W) to reduce viscosity. Samples were then incubated at 95^0C for 4 min and this represented the input sample. For the remainder of the suspension, cells were pelleted as described before and lysed in 1 ml of Buffer A (6 M Guanidine:Hcl, 10 mM Tris in phosphate buffer pH 8.0) supplemented with 5 mM imidazole, 50 mM iodoacetamide and 0.1 % (v/v) Triton X-100. After vigorous vortexing, the sample was sonicated as described earlier, before being added to 60 µl of bead-suspension which had been washed twice with 200 µl volumes of Buffer A. Thereafter, the mixture was incubated overnight at RT with end-over-end tumbling before the resin was pelleted (5000g, 1 min, RT). Supernatant was removed and the resin was washed with 0.8 ml of Buffer A supplemented with 0.1 % (v/v) Triton X-100, incubating at RT for 5 min with end-over-end rotation. Afterwards, three washing steps were carried out sequentially: i) 0.85 ml Buffer B (8 M Urea, 10 mM Tris in phosphate buffer pH 8.0) supplemented with 0.1 % (v/v) Triton X-100; ii) 0.9 ml Buffer C (8 M Urea, 10 mM Tris in phosphate buffer pH 6.5) supplemented with 0.2 % (v/v) Triton X-100 ; and iii) 0.95 ml Buffer C supplemented with 0.1 % (v/v) Triton X-100. After the completion of washing, to elute the material that remains bound to the resin, 50 µl of elution buffer was added to the final pellet of resin before being gently vortexed. The

mixture was left standing at RT for 20 min prior to incubation at 95^0C for 4 min. Resin was pelleted (16000g, 1 min, RT) and the supernatant was transferred to a new eppendorf. This represents the IP and can be stored at -20^0C for later analysis.

2.4.9 β-Gal activity Assay

During this thesis, the pcDNA3.1/V5His/LacZ expression plasmid containing the β-galactosidase (β-gal) gene was cotransfected, together with a reporter and/or expression constructs, into cells in order to control for transfection efficiency. To assay for β-gal activity, the cells were disrupted in a lysis buffer, centrifuged at 11, 000 × *g* and 4°C for 10 min, before the supernatants were assayed directly for β-gal activity using. A substrate mixture was prepared as follows: for every 1.32 ml β-gal solution (4mg/ml 0.1 M sodium phosphate buffer pH7.5) add 4.02 ml 0.1 M sodium phosphate buffer (0.082 M Na_2HPO_4 and 0.018 M NaH_2PO_4) and 60ul 100 × Mg solution (0.1 M $MgCl_2$ and 4.2 M β-mercaptoethanol).

2.4.10 Glutathione assay

Intracellular glutathione (GSH) levels were measured according to the method of Tietze{Tietze, 1969 #387}. RL-34 cells were grown in 60 mm dished at the density of 1×10^6 cells per dish and trypsinised. Ice-cold complete medium was added to inhibit the enzyme, and the single-cell suspension was centrifuged at 400 *g* for 2 min at 4°C. The cell pellet was resuspended in ice-cold PBS and again collected by

centrifugation at 400 *g* for 2 min at 4°C. Cells were resuspended in 200 µl of lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% NP40) and fractured by 3 freeze/thaw cycles in liquid nitrogen. Cell debris was removed by centrifugation at 16,000 *g* for 3 min at 4°C. Aliquot the soluble fraction and mix in an equal volume 10% (v/v) ice-cold sulphosalicylic acid (SSA) and incubated on ice for 20 min. The precipitated protein was removed by centrifugation at 16,000 *g* for 3 min at 4°C. Supernatant was kept for later analysis.

A GSH standard curve was prepared in 10% (v/v) SSA with concentrations of 6, 12, 24, 48, 60, 80 and 120 µM. Sample analysis was carried out in triplicate in a 96-well plate, with 10 µl of sample or standard added to each well, followed by 150 µl of assay mixture (1 mM DTNB, 0.34 mM NADPH in 150 mM $NaPO_4$ buffer, pH 7.5, containing 7.5 mM EDTA), and finally GSH reductase solution (10 µl of 10 U/ml in 150 mM $NaPO_4$ buffer pH 7.5 containing 7.5 mM EDTA). Absorbance of the reaction at 415 nm was measured every 15 sec for 5 min in SpectraMax (Molecular devices). GSH concentrations were derived from the standard curve. Protein concentrations were measured by BCA assay and GSH concentration expressed as nmol GSH/mg protein.

2.5 Animal Husbandry

Health of animals was monitored daily. Mice were kept in the animal unit for four weeks to allow them adapt to the environment. By the time the mice were 11 weeks

old, they were treated with phytochemicals as appropriate by gavage once a day for 4 consecutive days. Phytochemicals were dissolved in PBS as a suspension at the concentration of 50 mg/ml or 400 mg/ml. A suspension of 200 µl/kg appropriate suspension was given to mouse by gavage resulting in the final concentration being 10/80 mg of chemicals per 1 kg of animal weight. Blood and urine were collected every second day 2 hs after the treatment. On the fifth day, 24 h after the last treatment, mice were culled and tissues were excised and immediately washed in PBS and finally transferred to universals for snap freezing in liquid nitrogen and stored at -80°C.

3 Induction of NQO1 by polyphones in an Nrf2-ARE dependent pathway

3.1 Introduction

3.1.1 Dietary flavonoids present in the diet as aglycone or glycosides

As a subclass of polyphones, flavonoids and their polymers constitute a large class of food constituents that accounts for approximately two thirds of the polyphones in our diet. More than 4000 flavonoids have been identified. They all share a common structure, consisting of two aromatic rings (A and B rings) linked by 3 carbon atoms that are usually contained within an oxygenated heterocyclic ring (the C ring). The generic structure of a typical flavonoid is shown in Figure 3.1. The structural requirements for the antioxidant and free radical scavenging functions of flavonoids include a hydroxyl group at carbon position 3, a double bond between carbon position 2 and 3, a carbonyl group at carbon position 4 and polyhydroxylation of the A an B aromatic ring. Most flavonoids are present in nature as glycosides and other conjugates. The most ubiquitous flavonoids in fruit and vegetables are flavonols, and the two most representative chemicals are quercetin and kaempferol. Flavonols are usually present in glycosylated forms and the sugar moieties associated with them are often glucose or rhamnose, though other sugars may also be involved such as galactose arabinose, xylose or gucuronic acid. Flavones are much less common than

flavonols in fruit and vegetables and consist of chiefly glycosides of luteolin and apigenin. The group of flavonoids present in high concentration in citrus fruit is the flavanones, the main aglycones of which are narigenin, hespertin and eriodictyol. They are generally glycosylated by a disaccharide, at carbon atom position 7, with either a neohesperidose or a rutinose. In contrast with other flavonoids, flavanols are not glycosylated in foods. They exist as both a monomer form (catechins) and a polymer form (proanthocyanidins). Catechins are especially rich in tea, while procyanidins are present in a wide range of fruit and vegetables. Antocyanins exist in different chemical forms and are highly unstable in the aglycone form. While they exist in plants, their degradation is prevented by glycosylation and esterification (Manach *et al.*, 2004).

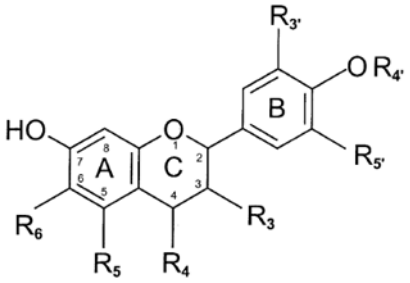


Figure 3.1 General structure and numbering nomenclature of flavonoids

3.1.2 Quercetin and kaempferol

The flavonols quercetin and kaempferol, the structures of which are shown in figure 3.2, are enriched in onions and apples (Miean & Mohamed, 2001). They exist as a variety of glycosides or in an aglycone form. Studies have shown that both the glycosides and the aglycone form are absorbed by the human gut though at different

efficiencies (Hollman & Katan, 1997). The aglycone forms of quercetin and kaempferol are similar in structure, differing only by one hydroxyl group in the B-ring. However, this one difference appears to account for the much higher free radical-scavenging activity of quercetin than kaempferol (Yamamoto *et al.*, 1999). The cancer preventive activity of quercetin has been extensively studied. In animal models, it has chemopreventive activity against tumourigenesis induced by PAHs (Mukhtar *et al.*, 1988). In cell culture models, quercetin exerts multiple biochemical effects that are relevant to carcinogenesis, including metal chelation, antioxidant properties, inhibition of hepatic enzymes, and the induction of drug-metabolizing enzymes. Induction of NQO1 by quercetin has been found in MCF-7 (Valerio *et al.*, 2001) and HepG2 cells (Tanigawa *et al.*, 2007). Epidemiological studies have shown that there is an inverse association between the intake of quercetin and lung cancer (Stefani *et al.*, 1999). Limited evidence has also been provided by Nöthlings that quercetin, as well as kaempferol, exerts a preventive effect on the development of pancreatic cancer in current smokers, but not in former and never smokers (Nothlings *et al.*, 2008). Another study showed that intake of kaempferol but not quercetin had an inverse association with the risk of ovarian cancer (Gates *et al.*, 2007). Taken together, it appears that quercetin and kaempferol can inhibit the initiation of cancers in various tissues.

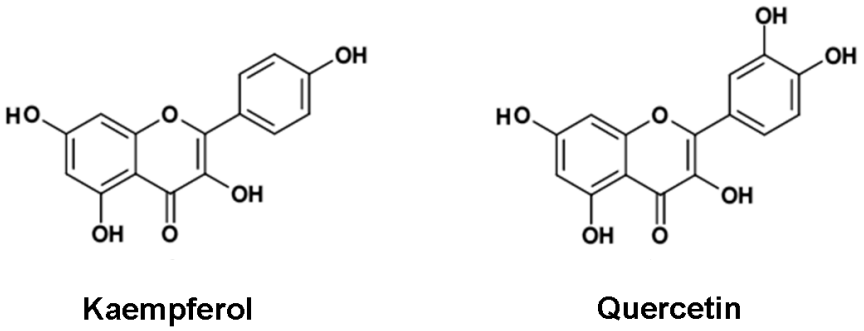


Figure 3.2 Structure of quercetin and kaempferol

3.1.3 Characterization of the AREc32 cells

A stable ARE-driven reporter gene cell line has been generated by Wang *et al.* (2006) that was derived from human breast carcinoma MCF7 cells. The reporter construct employed contained eight copies of the *cis*-element common to the rat *GSTA2* and mouse *Gsta1* gene promoters which had been ligated into the pGL3 promoter vector. Luciferase activity in AREc32 cell can be increased significantly by monofunctinal and bifunctional inducing agents including sulforaphane, t-BHQ, and β-naphthoflavone. Furthermore, the expression of ARE-driven luciferase activity in AREc32 cells is mediated by Nrf2. In the reporter cells, endogenous mRNAs for NQO1, GCLC, GCLM and AKR1C1 were increase by t-BHQ to various extents (Wang *et al.*, 2006).

3.1.4 Regulation of NQO1

NQO1 is a key enzyme involved in defence against oxygen reactive species and the inhibition of neoplasia. It can be induced upon oxidative stress and thus protects cells

against certain environmental insults such as benzo[α]pyrene produced from cigarette smoke and diesel exhaust. Expression of NQO1 is regulated in an Nrf2-ARE dependent fashion. The AREs identified in the regulatory region of the rat, mouse and human *NQO1* genes show conservation of the nucleotide sequence immediately adjacent to the 'core' enhancer. However, there is an important difference, between the ARE enhancers in the mouse, rat and human *NQO1*, in that the human *cis*-element contains an embedded AP-1 site (Jaiswal, 1991) which is absent from the AREs in the rodent genes (Favreau & Pickett, 1991; Nioi *et al.*, 2003). The Nrf2 CNC-bZIP transcription factor is recruited to the ARE of NQO1 as an obligate heterodimer with small Maf proteins (Itoh *et al.*, 1997; McMahon *et al.*, 2001; Nioi *et al.*, 2003; Thimmulappa *et al.*, 2002). Disruption of the mouse *Nrf2* gene not only abolishes the inducible expression of Nqo1 but also results in a reduction in its constitutive expression.

Besides the ARE in *NQO1*, a xenobiotic responsive element (XRE) has also indentified in the promoter region of the mouse, rat and human genes (Favreau & Pickett, 1991; Jaiswal, 1991; Nioi & Hayes, 2004). The XRE has the consensus sequence 5′-$T^{A}/_{T}$GCGTG-3′ and the adjacent 3′ nucleotide is often an A or a C, thus the 'core' enhancer is considered as 5′- $T^{A}/_{T}$GCGTG$^{A}/_{c}$-3′. The core sequence of XRE in mouse and rat *Nqo1* are closely similar. In mouse *Nqo1*, the core sequence of the XRE in *NQO1* is 5′-TAGCGTGC-3′, between -386 and -379 from the transcriptional start site, located 41 bp upstream of the 'core' ARE. In the rat *Nqo1*

gene, a closely similar 5′-TTGCGTGC-3′ sequence is located between -372 and -365 nucleotide from the transcriptional start site, 40 bp downstream of the 'core' ARE. By contrast, the XRE in human *NQO1*, 5′-AGGCGTGA-3′, between -742 and -735, is located 274 bp upstream of its 'core' ARE. In addition, the core XRE in rodent *Nqo1* shared close similiarity with that in rodent *Cyp1a1*. Induction of Nqo1 enzyme activity by TCDD has been observed in Hepa-1c1c7 cells but not in Hepa-1c1c7 cells that possess defects in AhR or Arnt (Ma *et al.,* 2004). These findings suggest that Nqo1 can be activated by chemicals that are AhR ligands. Furthermore, using $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse embryonic fibroblasts, it has been found that induction of NQO1 mRNA by TCDD is Nrf2-dependent (Ma *et al.,* 2004). These findings suggest that NQO1 can be regulated by the AhR through the XRE and by Nrf2 through the ARE upon exposure to different chemicals. Moreover, there may be some type of cross-talk between the AhR and Nrf2 transcription factors.

3.1.5 $Nrf2^{+/+}$, $Nrf2^{-/-}$ and DBA/2O MEF cells

The $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse embryonic fibroblasts were derived from mice on a C57BL/6 genetic background. The $Nrf2^{-/-}$ MEFs were derived from homozygous mutant mice, in which the b-ZIP region of the *Nrf2* gene was replaced with a recombinant SV40 nuclear localization signal (NLS)-*β*-galactosidase *(lacZ*) recombinant gene (Itoh *et al.*, 1997). The DBA/2 inbred mice are regarded as being genetically "nonresponsive" when compared with "responsive" strains such as the C57BL/6 mouse. The later animals exhibit high induction of CYP1A1 enzyme

activity in liver when they are treated with nonhalogenated polycyclic aromatic hydrocarbons such as MC, BP, or BA. By contrast “nonresponsive” mice do not show a significant increase in hepatic P-450 even when treated with very high doses of nonhalogenated PAHs. For the highly potent halogenated inducer TCDD, though it can induce P-450 in “nonresponsive” strains of mice, the dose required for half-maximal induction by TCDD is approximately 15-fold higher in DBA/2 mice than in C57BL/6 mice. In addition, though AhR receptors were found to be present in the cytosol of liver of DBA/2 mice, they have very low affinity to the ligands (Okey *et al.*, 1989). In 1993, Chang *et al*. found the Ah receptor locus polymorphism of C57BL/6 and DBA/2 mice are due to the amino acid changes between the two different allelic forms of AhR in C57BL/6 and DBA/2O. This research group discovered there were a total of ten nucleotide differences between the two alleles, five of which were responsible for amino acid substitutions, leading to the structural change. Such structural changes possibly result in the different affinity of AhR in the two different mouse strains (Chang *et al.*, 1993).

3.1.6 Aim

Selected flavonoids, the ones distributed in commonly consumed fruits and vegetables, were screened using AREc32 cells to find the most potent ARE-inducers. Subsequently, experiments were carried out to see whether these phytochemicals increase the expression of NQO1 and whether it is Nrf2 dependent. Furthermore the involvement of the ARE and XRE enhancers in the promoter region of mouse *Nqo1*

in gene induction by phytochemicals was also investigated using mutagenesis analysis. Finally, the biological consequence of gene induction by flavonoids was examined to see whether they could provide protection against cytotoxicity induced by acrolein.

3.2 Results

3.2.1 Screen to identify phytochemicals that induce ARE-driven gene expression using the AREc32 reporter cells

3.2.1.1 Determination of the optimal time interval required for ARE-driven gene induction.

The AREc32 reporter cell line was used to screen the ability of polyphenols to induce ARE-driven gene expression. To determine the optimal induction of luciferase activity, a time course experiment was carried out using the isothiocyanate sulforaphane, which is a classic activator of ARE-driven genes. AREc32 cells were treated with sulforaphane at a final concentration of 10 μmol/l for various periods of time as indicated in Fig 3.3. It was found that upon treatment with sulforaphane, luciferase activity increased with time, reaching a maximum of around 13-fold at 24 hours. Though we tried 48 hours as well, the medium dried out and this is not technically applicable for 96-well plate format. Thus AREc32 cells were treated for 24 hours with the test polyphenols in the luciferase reporter activity screening

experiment. In addition, sulforaphane was included as a positive control in every plate of each experiment.

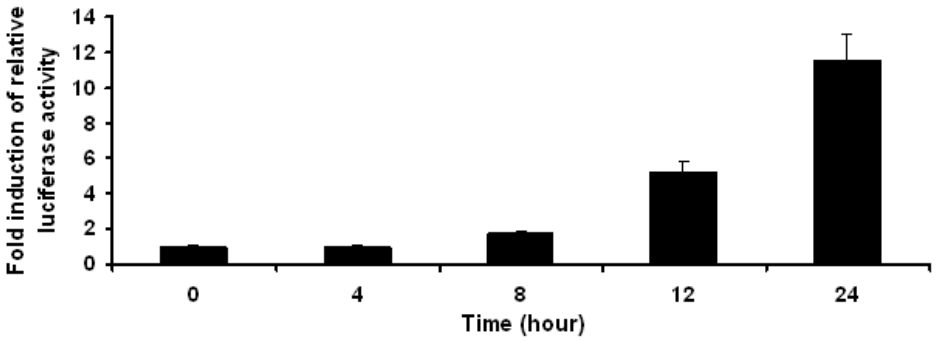


Figure 3.3 Time dependent induction of luciferase activity in AREc32 cells by sulforaphane
AREc32 cells were treated with sulforaphane (10 μmol/l) for different periods of time before being harvested simultaneously and luciferase activity measured. Experiments were performed on at least three independent occasions. Data represent mean ± standard deviation.

3.2.1.2 Screening of polyphenols to identify ARE-inducers

The flavonoids selected for study include flavonols, flavanones, catechin tannins and anthocyans. The flavonol group contained: quercetin and its glycosides quercitrin, rutin and quercetin-3-*O*-glucopyranoside; kaempferol and its glycosides kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside; the flavanone group consisted of narigenin, its glycoside narigenin-7-*O*-glucoside, and the glycoside hesperitin; the catechin tannin group included (-)-epicatechin and (-)-epigallocatechin gallate; the anthocyan group included cyanidin chloride and its glycosides kuromanin chloride and keracyanin chloride; callistephin chloride, oenin chloride and myritillin chloride. The AREc32 cells were treated with the phytochemicals at a concentration of 20 μmol/l for 24 hours before luciferase activity was measured. Each treatment was carried out in triplicate and every experiment was

performed on at least three separate occasions. The results are shown in Figure 3.4. Amongst these compounds, quercetin and kaempferol from the flavonoid family stimulated the highest induction, producing increases of 4.5-fold and 3.8-fold, respectively ($p < 0.001$). It is interesting to note that the glycosides of quercetin and kaempferol did not produce comparable increases in luciferase activity, though rutin gave a 10% induction ($p < 0.05$). Besides quercetin and kaempferol, narigenin, a flavanone gave a 30% ($p < 0.05$) increase in luciferase activity. From the anthocyan family, aglycone caynidin chloride increased reporter gene activity 17% while its glycosides did not appear to stimulate any significant induction; another two glycosides keracyanin chloride and oenin chloride gave inductions of 34% and 26% respectively. No effect on the luciferase activity was observed for the chemicals from the catechin tannin family. As these chemicals were used at a concentration of 20 μmol/l, which is much higher than the physiological concentration, a 2-fold induction was considered worth pursuing. On this basis, the two most potent phytochemicals quercetin and kaempferol from the flavonol family were used for further studies.

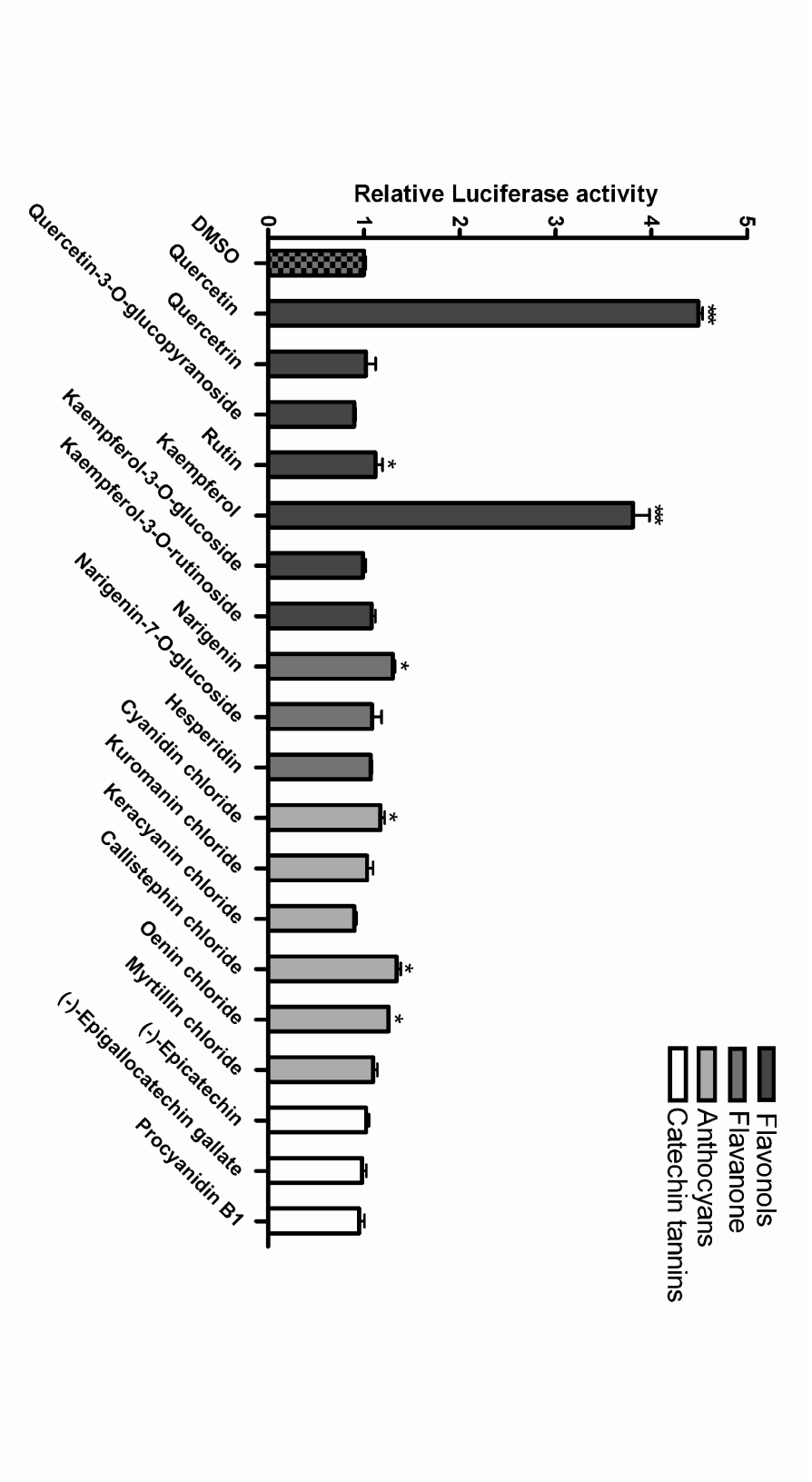


Figure 3.4 Preliminary screening using AREc32 cells.

All chemicals were used at a final concentration of 20 μmol/l. Cells were treated with these chemicals for 24 h before luciferase activity was measured. Experiments were performed on at least three independent occasions. Data represent mean ± standard deviation.

3.2.1.3 Dose-response of gene induction by quercetin and kaempferol

A dose-response experiment was carried out for quercetin and kaempferol to determine the maximal concentration of polyphenol required to induce ARE-drive gene expression. As shown in Figure 3.5, both quercetin and kaempferol were able to induce ARE-driven luciferase activity in a dose-dependent manner. Their ability to induce luciferase activity reached a plateau at concentrations of between 10 and 20 μmol/l. Thus a final concentration of 20 μM quercetin and 20 μM kaempferol was chosen for subsequent experiments.

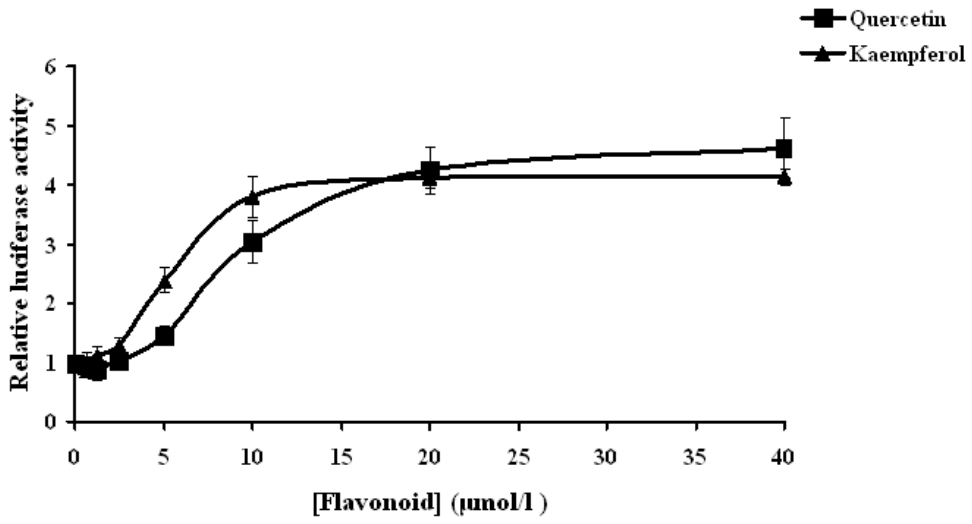


Figure 3.5 Dose-dependent induction of reporter gene activity in AREc32 cells by quercetin and kaempferol.

AREc32 cells were treated with either quercetin or kaempferol at various concentrations for 24 h before luciferase activity was measured. Experiments were performed on at least three independent occasions. Data represent mean ± standard deviation.

3.2.2 Design and use of XRE luciferase reporter plasmids

A pGL_4×XRE-Luc luciferase reporter construct was generated as described in

2.2.2.3. It was transfected into RL-34 cells along with β-Gal. After 18 h recovery from transfection, the cells were treated for 24 h with 1 μM β-NF or 1 μM ICZ. Thereafter, luciferase activity was measured as described in section 2.2.5. The result in Figure 3.6 showed that even treatment with the typical XRE inducers β-NF or ICZ gave increase in reporter activity of only 1.4- and 1.5-fold, respectively, which suggests that this construct is not responsive enough in RL-34 cells. It therefore was not used to screen XRE inducers.

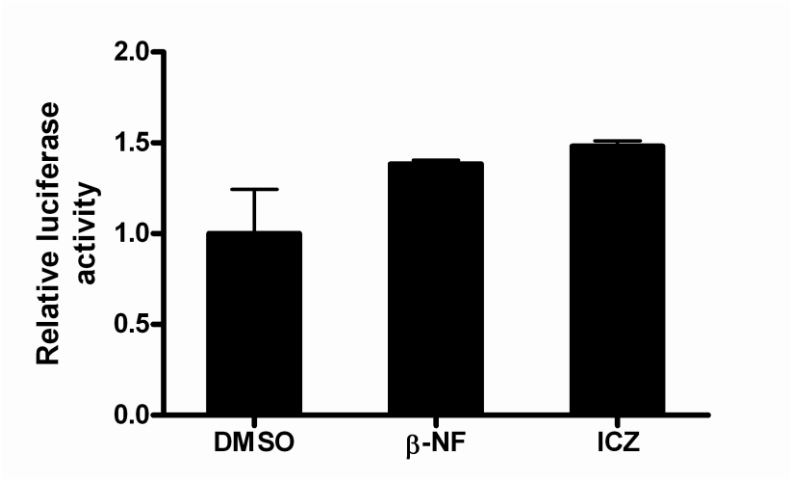


Figure 3.6 Performance of the pGL_4×XRE-Luc reporter plasmid.
RL-34 cells were transfected with pGL_4×XRE-Luc along with β-Gal. After transfection, cells were treated with β-NF (1 μmol/l) or ICZ (1 μmol/l) for 24 h before luciferase activity was measured. Experiments were performed for at least three independent occasions. Data represent mean ± standard deviation.

3.2.3 Phytochemicals have no effect on the expression of GSTP1 protein in RL-34 cells

Having shown that quercetin and kaempferol can induce ARE-driven gene expression, we examined their effect on the expression of GSTP1 because its gene is thought to contain a functional ARE. Non-transformed rat liver RL-34 cells were

seeded in 60 mm dishes. After they reached 80% confluence, they were treated with phytochemicals for 24 h. Whole cell lysates were analysed for the level of GSTP1 protein in the cells. As shown in Figure 3.7, neither quercetin nor kaempferol had any effect on the expression of GSTP1 protein.

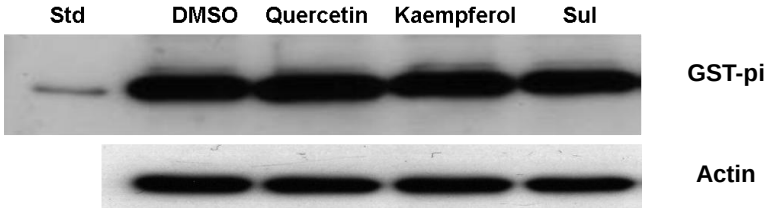
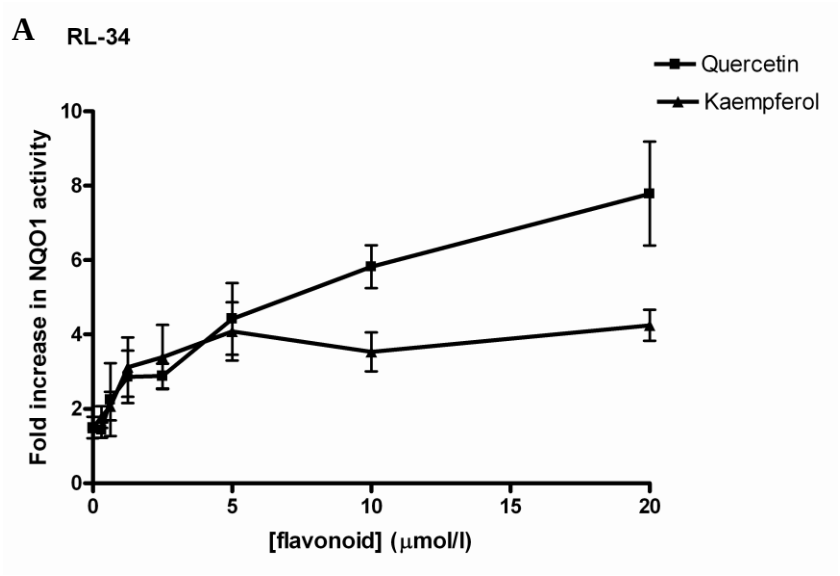


Figure 3.7 No effect by quercetin or kaempferol on the expression of GSTP1.
RL-34 cells were treated with quercetin or kaempferol at the final concentration of 20 μmol/l and sulforaphane of 5 μmol/l. The same blot was stripped and re-probed with anti-actin antibody.

3.2.4 Quercetin and kaempferol increase NQO1 enzyme activity in a dose-dependent manner

Having shown that quercetin and kaempferol induce reporter gene expression in the AREc32 cells, we examined their effect on NQO1. For this purpose, non-transformed rat liver RL-34 cells and primary MEF cells were used. Firstly the effect of quercetin and kaempferol on NQO1 enzyme activity was examined. Cells were treated for 24 h with quercetin, kaempferol or DMSO before NQO1 activity was measured. Both quercetin and kaempferol increased the oxidoreductase activity in a dose-dependent manner in both cell lines (Figure 3.8). In RL-34 cells, quercetin increased NQO1 activity 4.5-fold at the highest concentration used, which was 20 μmol/l. By contrast, kaempferol increased NQO1 activity 2.8-fold at the same

concentration. In *Nrf2*$^{+/+}$ MEF cells, quercetin and kaempferol increased the NQO1 activity 2.2-fold and 1.3-fold, respectively, at a concentration of 20 μmol/l. When the experiment was performed in *Nrf2*$^{-/-}$ fibroblasts cells, the basal NQO1 enzyme activity was substantially lower than was observed in *Nrf2*$^{+/+}$ fibroblasts, and no increase was observed upon treatment with either quercetin or kaempferol. These results suggest that Nrf2 regulates both basal NQO1 activity and its induction by quercetin and kaempferol.



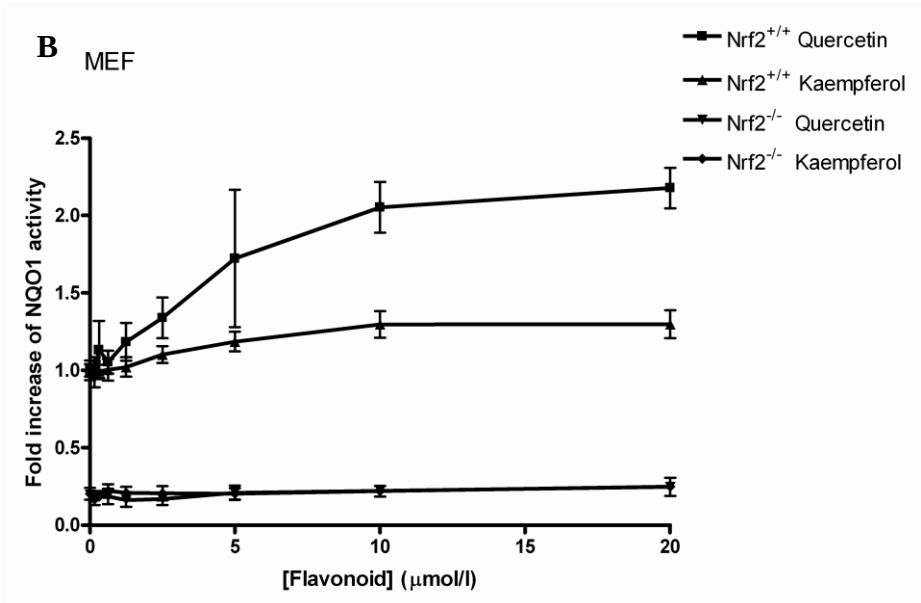


Figure 3.8 Quercetin and kaempferol increase NQO1 enzyme activity in a dose-dependent and Nrf2-dependent manner.

RL-34 (A), $Nrf2^{+/+}$ and $Nrf2^{-/-}$ MEF cells (B) were treated with various concentrations of quercetin or kaempferol for 24 h before the enzyme activity was measured as described in Materials and Methods. The value of enzyme activity was normalized to that of protein concentration and treated cells were compared with untreated cells.

3.2.5 Induction of NQO1 by phytochemicals

As treatment with quercetin or kaempferol increased NQO1 enzyme activity, we examined whether they could increase the level of the oxidoreductase protein. Thus endogenous NQO1 protein in RL-34 and MEF cells was measured by Western blotting following treatment with quercetin or kaempferol at a concentration of 20 μmol/l for 24 hours; in addition, sulforaphane (5 μmol/l), a typical activator of Nrf2, and 3-MC (1 μmol/l), a typical AhR agonist, were used as positive controls. Western blotting showed that both quercetin and kaempferol increased the endogenous NQO1 protein in RL-34 and *Nrf2*$^{+/+}$ MEF cells (Figure 3.9). Densitometry analysis in Figure 3.9 A showed that quercetin increased NQO1 protein approximately 30-fold in RL-34 cells whereas kaempferol increased NQO1 protein levels about 25-fold in the same cell line. Both flavonoids increased Nqo1 protein in MEF cells, as shown in Figure 3.9B, to a much lesser extent than was observed in RL34 cells; quercetin increased the amount of Nqo1 protein around 3-fold while kaempferol produced a 1.5-fold increase. Furthermore, the basal level of Nqo1 protein and its level of induction were substantially lower in $Nrf2^{-/-}$ MEF cells than in wild-type fibroblasts. It was also found that treatment with 3-MC produced a moderate increase in Nqo1 protein of about 10-fold in RL-34 cells, but this was not observed in either wild-type

or Nrf2 knockout MEF cells.

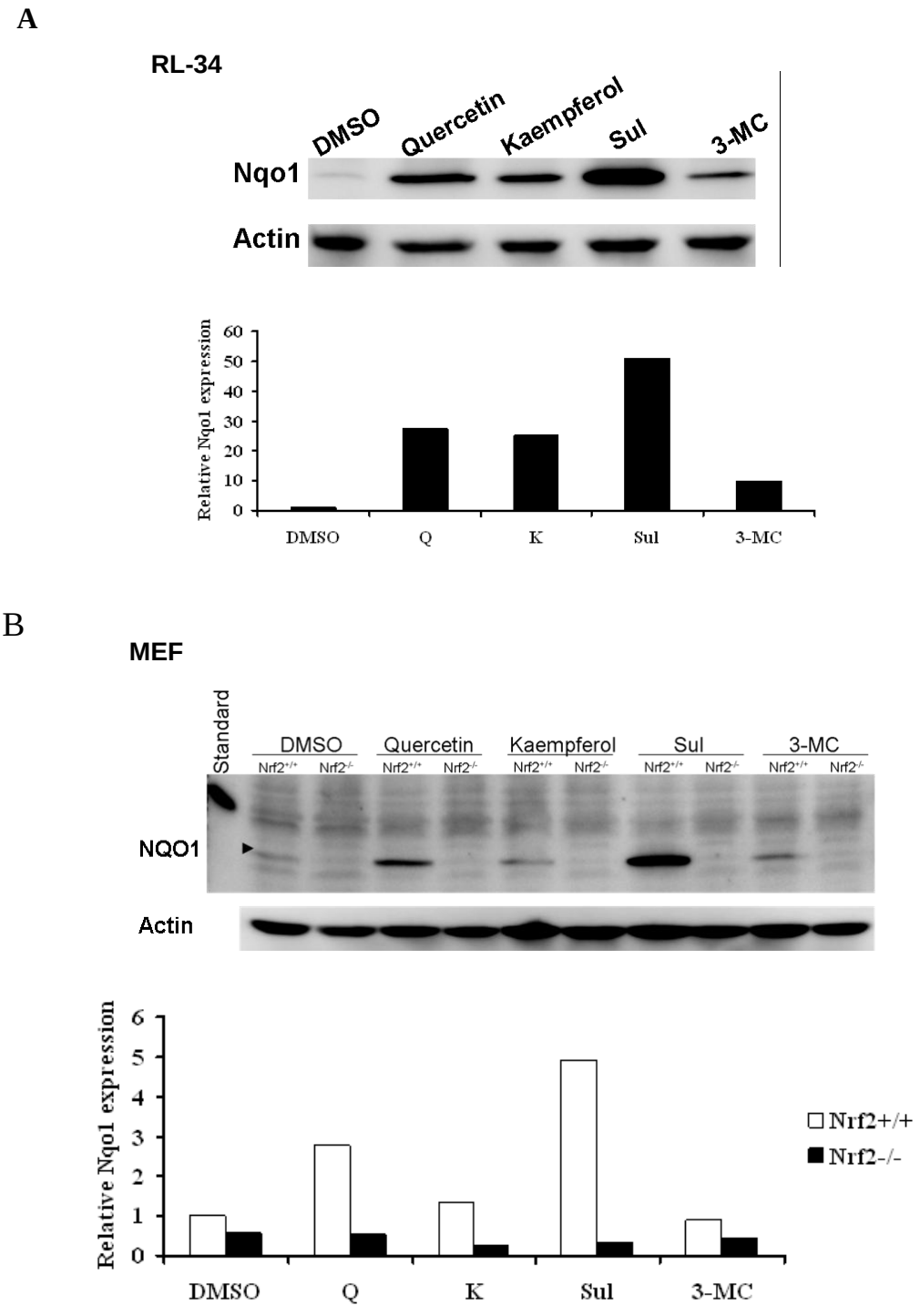


Figure 3.9 Phytochemicals increase the level of Nqo1 protein in rodent cells in an Nrf2-dependent manner.

RL-34 (A), *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ MEF cells (B) were seed in 60 mm dishes. After ~ 24 h recovery, when cells reached ~80% confluence, they were exposed to vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l), sulforaphane (5 μmol/l) or 3-MC (1 μmol/l). Protein preparation and detection of Nqo1 protein by Western blotting were performed as described in Materials and Methods.

After blotting for Nqo1, the membranes were stripped and re-probed with actin. The right hand panel of (A) and lower panel of (B) are the densitometry analysis of the immunoblots. The values of the treated cells were compared with untreated cells. For MEF cells all values were compared with that of untreated $Nrf2^{+/+}$ MEF cells.

3.2.6 Treatment with flavonoids increases *Nqo1* mRNA in RL-34 and MEF cells

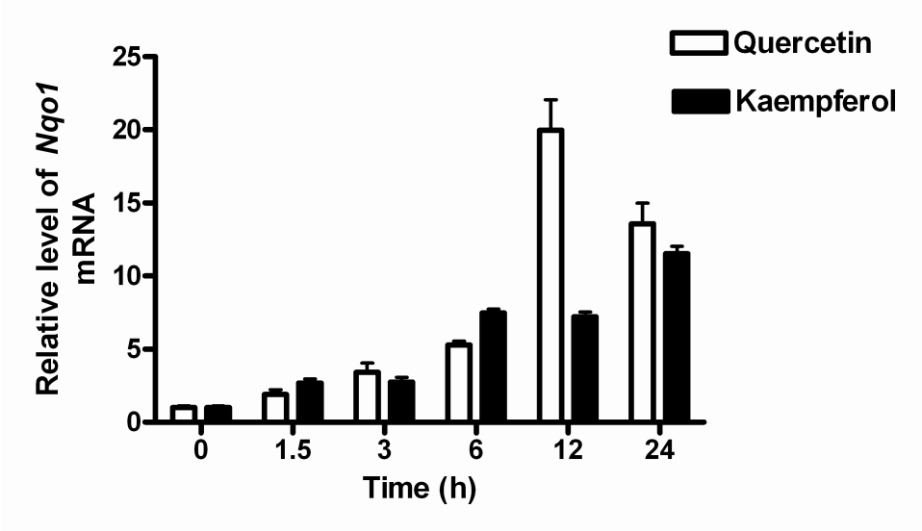
3.2.6.1 Change of mRNA of Nqo1 by quercetin or kaempferol over time

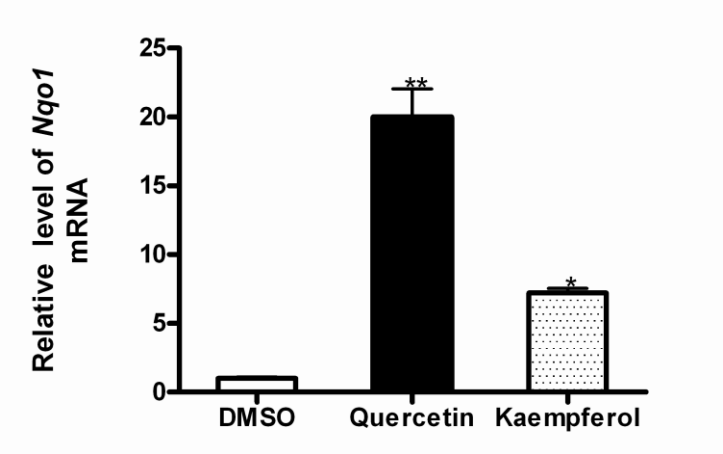
After showing that quercetin and kaempferol can increase Nqo1 protein levels, we further investigated whether they regulated Nqo1 at the transcriptional level. Cells were exposed to quercetin, kaempferol or DMSO for various times as indicated in Figure 3.10. Total mRNA was extracted and that for Nqo1was measured by qT-PCR. The time course experiment showed that in RL-34 cells (figure 3.10 A), quercetin induced Nqo1 mRNA in a time dependent manner with the increase reaching a maximum of around 20-fold at 12 hours. Thereafter, the amount of Nqo1 mRNA decreased to around 13-fold at 24 hours. Though kaempferol also induced Nqo1 mRNA over time, the increase appeared more gradual and sustained than was observed for quercetin.

In $Nrf2^{+/+}$ MEF cells (figure 3.10 B), both quercetin and kaempferol increased Nqo1 mRNA in a time dependent manner giving the highest induction at 12 hour of approximately 10-fold and 6-fold, respectively. Thereafter, the induction decreased gradually and was estimated to be 5-fold and 3-fold for quercetin and kaempferol, respectively, at 24 h. In $Nrf2^{-/-}$ MEF cells (Figure 3.10 B), the basal level of Nqo1

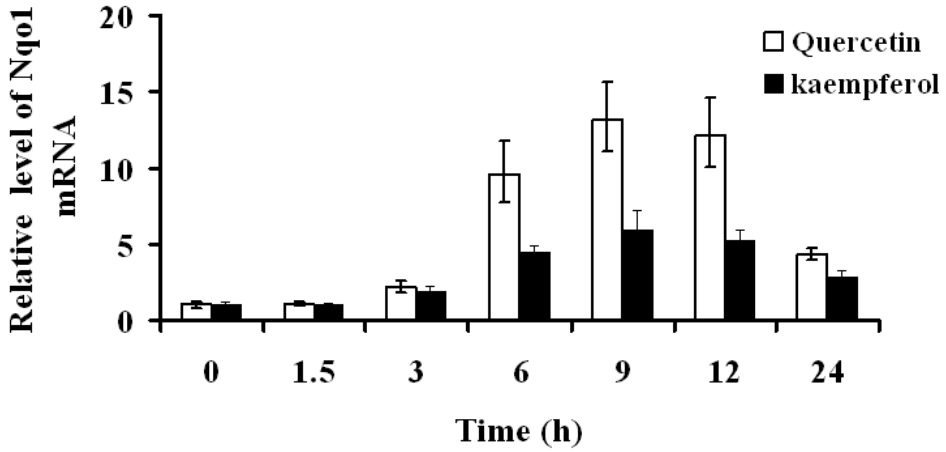
mRNA was substantially lower than in wild-type fibroblasts. The induction level produced by quercetin and kaempferol in *Nrf2*$^{-/-}$ MEF cells was around 3-fold and 2.5-fold respectively. Collectively, these results show that both quercetin and kaempferol can increase Nqo1 enzyme activity, as well as its expression at both the protein and mRNA levels and these increases were principally Nrf2-dependent, but a small Nrf2-independent increase was also observed. In addition, quercetin was more potent than kaempferol as an Nqo1 inducting agent in both of the cell lines examined.

A





B



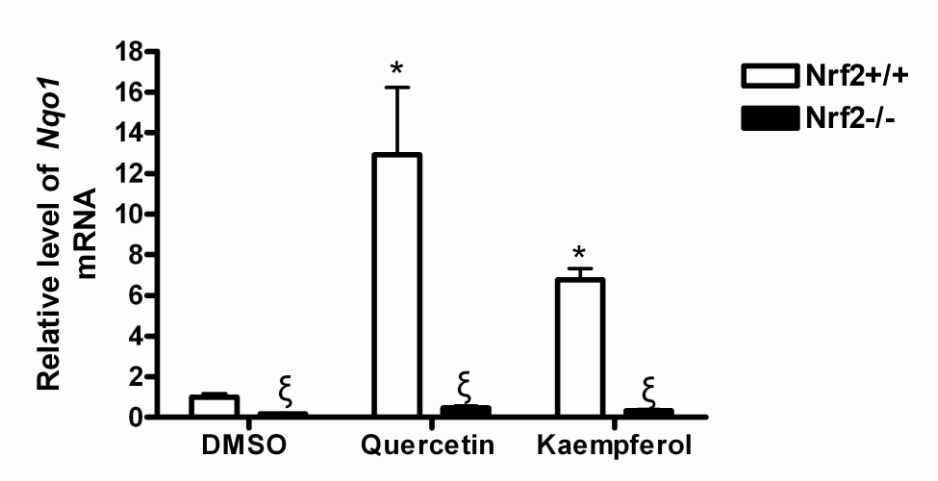


Figure 3.10 Quercetin and kaempferol increase the mRNA level of Nqo1 in an Nrf2-dependent manner.

RL-34 (A), *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ MEF cells (B) were seeded in 60 mm dishes and left to recover for ~24 h to reach ~80% confluence. Cells were then treated with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l), for various time points and harvested simultaneously. RNA was then extracted and reverse transcribed to cDNA as described in Materials and Methods. cDNA was then used for Taq-man to detect the amount of *Nqo1* mRNA. At least three independent experiments were performed. Data represented the mean ± standard error. The lower panel in (A) and lower panel in (B) represent the data at 12 time point. Treated cells were compared with DMSO treated cell (DMSO treated Nrf2$^{+/+}$ MEF cells for MEF cells). Statistical analysis was carried out and student's t-test was carried out for the 12 time point value. (**/ξξ, P< 0.01; */ξP<0.05; * indicates postivite change while ξ indicates negative change)

3.2.6.2 Consecutive treatments with quercetin or kaempferol

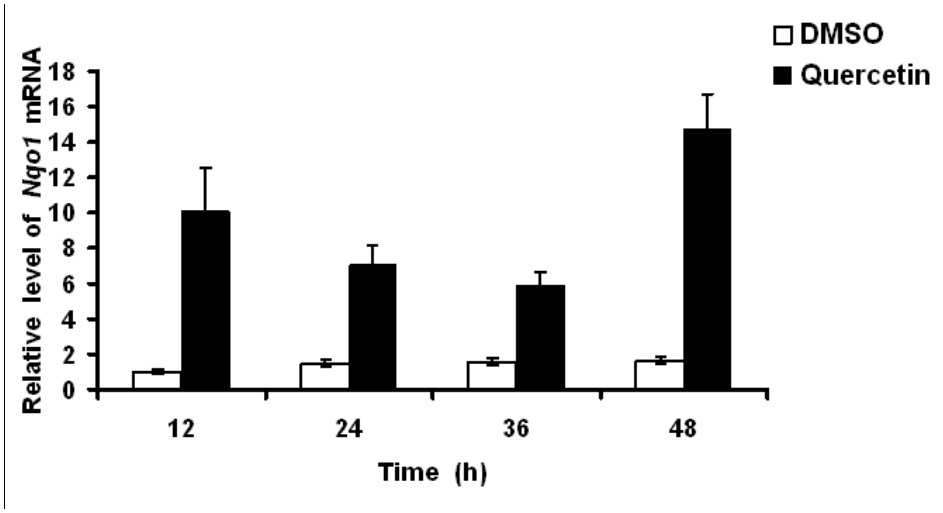
After showing that both quercetin and kaempferol can induce the Nqo1 mRNA level, we wished to know whether re-treatment of RL-34 and MEF cells with quercetin or kaempferol could maintain elevation of the oxidoreductase over an extended period of time. As the time course showed that quercetin elicits the highest induction at 12 h in both RL-34 and MEF cells, whereas kaempferol showed highest induction at 24 h or 12 h in RL-34 or MEF cells respectively, RL-34 and $Nrf2^{+/+}$ MEF cells were treated with quercetin or kaempferol every 12 h for 12, 24, 36 or 48 h to determine whether repeated treatment with polyphenols caused a sustained increase in Nqo1. To ensure that all cells for the polyphenol repeated treatment experiment were harvested at the same time, they were first exposed to the flavonoids at different time points. Thereafter, mRNA was extracted, reverse transcribed to cDNA and measured by Taq-man. The analysis in figure 3.11 A showed that in RL-34 cells the increase in NQO1 mRNA by quercetin dropped gradually from 10-fold at 12 h to 5-fold and 4-fold at 24 h and 36 h, respectively, while the induction went diminished to 9-fold at 48 h. On the other hand, the increase in Nqo1 mRNA by kaempferol was more stable over time with moderate decrease from 7-fold at 12 h to 4.3-fold at 24 h, going back at 5.5-fold and slightly dropped down again at 48 h to 4.5-fold. However, the change from 12 h to 24 h and followed by that in 48 h was not significant.

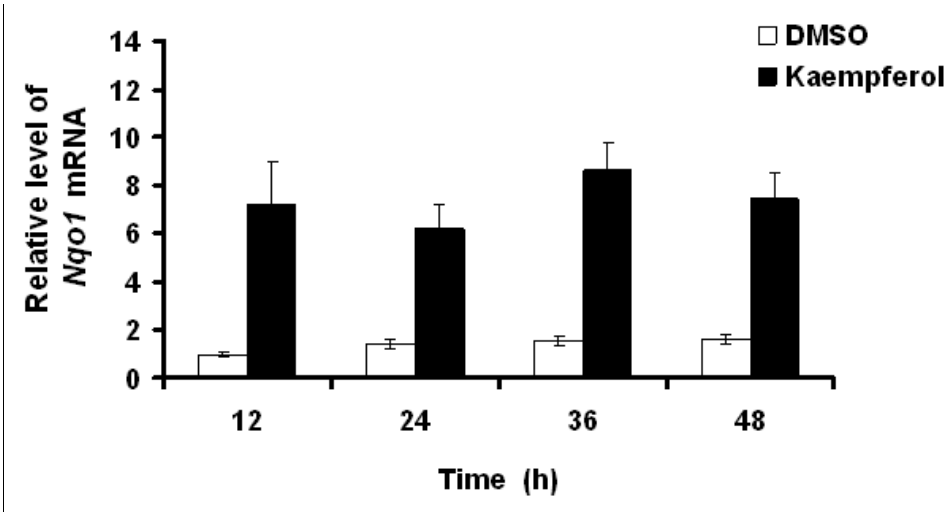
The pattern of induction of NQO1 mRNA by flavonoids in MEF cells (figure 3.11 B) differed from that observed in RL-34 cells. The increase of Nqo1 mRNA by

quercetin appeared similar at 12 h and 24 h, which was around 11-fold, but it decreased thereafter from 7-fold at 36 h to around 3-fold at 48 h. In the case of kaempferol, increase in Nqo1 mRNA dropped from 10.5-fold at 12 h to 3.7-fold at 24 h and kept decreasing further over time, maintaining an induction of around 3-fold at 36 and 48 h. It was interesting to notice that over time the basal level of NQO1 mRNA increased in RL-34 cells but decreased in MEF cells.

A

RL-34





B

MEF

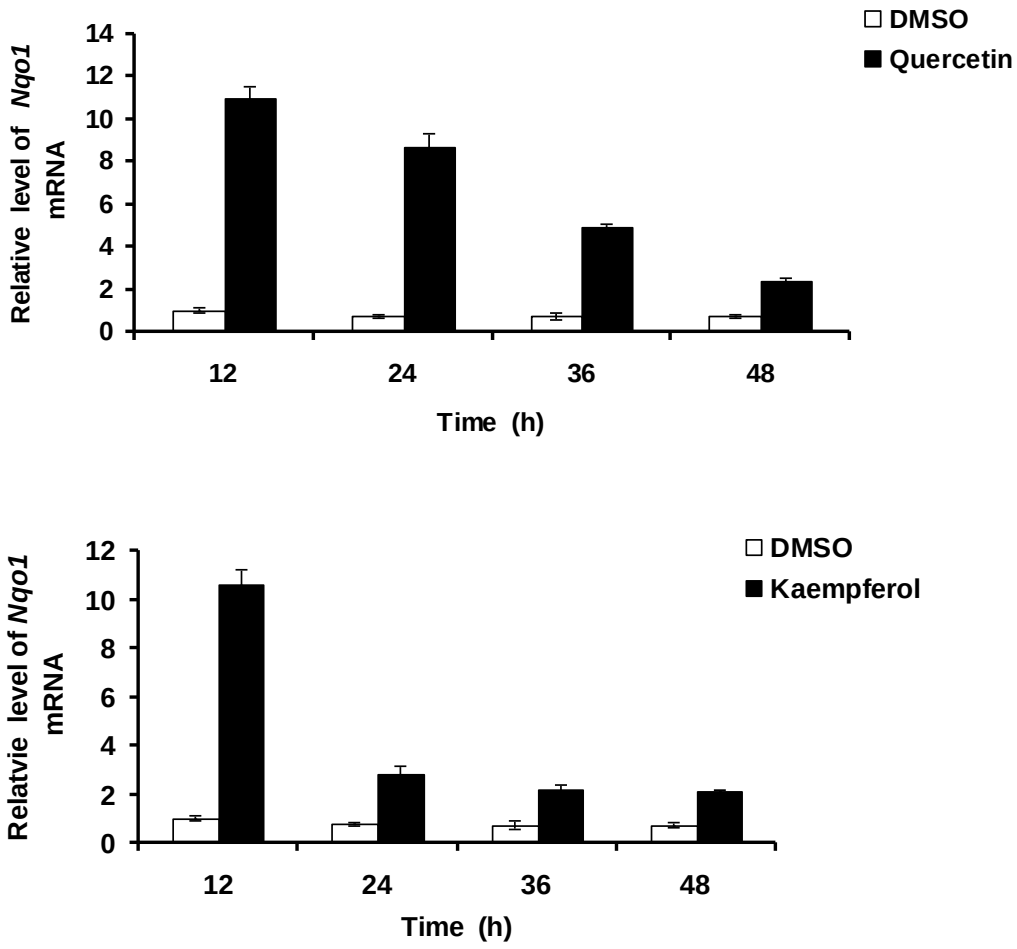


Figure 3.11 Consecutive treatment with quercetin and kaempferol affected the mRNA level differently in RL-34 and Nrf2$^{+/+}$ MEF cells.

RL-34 (A) and *Nrf2*$^{+/+}$ MEF cells (B) were seeded in 60-mm dishes and left to recover for ~24 h. When cells were ~70% confluent, they were either treated for 12, 24, 36, or 48 h with polyphenol. For each length of treatment, cells were washed and re-cultured in growth media containing either vehicle DMSO (0.1, v/v), quercetin (20 μmol/l) or kaempferol (20 μmol/l). Cells were harvested for analysis at the same time. Measurement of *Nqo1* mRNA was carried out as described in Figure 3.10. All values were compared with that observed when cells were treated with DMSO for 12 h.

3.2.7 Introducing mutations to ARE or XRE in Nqo1-luciferase reporter constructs

The upstream regulatory region of mammalian *Nqo1* genes contains both ARE and XRE enhancer sequences (Nioi & Hayes, 2004). Although it has been well

documented that Nqo1 is induced by sulforaphane and tBHQ through an ARE sequence, in an Nrf2-dependent manner, it was not known whether quercetin and kaempferol induced NQO1 through the ARE or XRE, or through both cis-elements. To address this issue, a series of Nqo1-luciferase reporter constructs were made. As described in section 2.2.2.1, using $P_{-1016/nqo1}$-Luc as a template, three constructs containing the transversion mutation in the ARE, XRE or ARE and XRE were made and named as $_{mut}ARE_{-1016/nqo1}$-Luc, $_{mut}XRE_{-1016/nqo1}$-Luc or $_{mut}ARE/_{mut}XRE_{-1016/Nqo1}$-Luc. RL-34 cells or MEF cells were transfected with either of these constructs along with pcDNA4/HisMax/lacZ encoding β-galactosidase (β-gal). After transfection, cells were treated with various chemicals and luciferase activity was measured and normalized to β-gal activity. As shown in Figure 3.12, when RL-34 cells, that had been transfected with $P_{-1016/NQO1}$-Luc, were treated with 20 μmol/l quercetin or 20 μmol/l kaempferol, an induction of reporter gene activity of around 4- or 3.8-fold, respectively, was observed. By comparison, Sul or ICZ at a concentration of 5 μmol/l or 1 μmol/l, respectively, gave an induction of around 4.5- and1.9-fold induction, respectively. When the ARE in the $P_{-1016/Nqo1}$-Luc reporter construct was mutated, both the basal level and induction level for all chemicals were dramatically decreased. When the XRE was mutated, basal reporter activity level decreased to 16% and the levels of induction produced by quercetin, kaempferol, Sul and ICZ were decreased to various degrees with ICZ showing the greatest reduction. Unexpectedly, when cells were transfected with $_{mut}ARE/_{mut}XRE_{-1016/nqo1}$-Luc, basal reporter activity was decreased to 25% compared with cells transfected with

$P_{-1016/nqo1}$-Luc; however, it was higher than that from constructs containing mutation in just the ARE or XRE alone.

The responsiveness of the Nqo1-lucifersase constructs to flavonoids was examined in *Nrf2*$^{+/+}$, *Nrf2*$^{-/-}$, and DBA/2 MEF cells (Figure 3.13). Treatment of mouse wild-type fibroblasts, which had been transfected with the wild-type reporter constructs, with quercetin or kaempferol for 24 h resulted in an increased luciferase activity of around 2-fold. When cells were transfected with $_{mut}ARE_{-1016/nqo1}$-Luc, both the basal and inducible luciferase activities were dramatically decreased. By contrast, when cells were transfected with $_{mut}XRE_{-1016/nqo1}$-Luc, no significant change was observed in the basal luciferase activity or induction of luciferase activity by quercetin; however the induction level by kaempferol was decreased to 1.4-fold. When cells were transfected with $_{mut}ARE/_{mut}XRE_{-1016/nqo1}$-Luc, basal luciferase and inducible activity were comparable with the results obtained when the ARE was mutated (Figure 3.13 A).

Taken together, the results from RL-34 and *Nrf2*$^{+/+}$ MEF cells indicated that both the basal activity from the Nqo1-luciferase reporters and the level of induction produced by quercetin and kaempferol are dictated by the ARE. To determine whether the basal and inducible reporter activity was mediated by Nrf2, we performed the same experiment in *Nrf2*$^{-/-}$ MEFs (Figure 3.13 B). This showed that the mutant fibroblasts transfected with $P_{-1016/nqo1}$-Luc produced substantially lower basal and inducible

luciferase activity when treated with flavonoids, compared with wild-type fibroblasts. When cells were transfected with $_{mut}$ARE$_{-1016nNqo1}$-Luc or $_{mut}$XRE$_{-1016/nqo1}$-Luc, both the basal expression flavonoids induced expression were decreased slightly compared with in *Nrf2*$^{-/-}$ MEFs transfected with the P$_{-1016/nqo1}$-Luc. When *Nrf2*$^{-/-}$ MEFs were transfected with $_{mut}$ARE/$_{mut}$XRE $_{-1016/nqo1}$-Luc, there was a small decrease in the basal level but this was not significant. These results suggested that Nrf2 plays an essential role in the basal expression of Nqo1 and its induction by quercetin and kaempferol.

To investigate whether the XRE is involved in either basal or inducible Nqo1 regulation, the same experiment was carried out using DBA2/O MEF cells in which AhR has very low ligand binding affinity. As *Nrf2*$^{+/+}$ MEF (C57BL/6) and DBA2/O MEF cells were generated from mice with different genetic background, the basal levels of NQO1 were not comparable between these two cells lines. However, we wanted to see whether the loss of AhR function can affect the level of induction produced by flavonoids. When the DBA/2O fibroblasts were transfected with P$_{-1016/nqo1}$-Luc, the induction of reporter gene activity by quercetin was lower than that observed in *Nrf2*$^{+/+}$ MEF cell (Figure 3.13 C). However, in the case of kaempferol, higher luciferase activity was detected in DBA/2O MEF than in *Nrf2*$^{+/+}$ MEF. When the DBA2/O fibroblasts were transfected with $_{mut}$ARE$_{-1016/nqo1}$-Luc, the situation was the same as that in *Nrf2*$^{+/+}$ MEFs. When cells were transfected with $_{mut}$XRE$_{-1016/nqo1}$-Luc, basal luciferase activity was decreased by 30% and induction by

quercetin did not change significantly compared with that observed when they were transfected with $P_{-1016/nqo1}$-Luc. By contrast, when cells were treated with kaempferol, luciferase activity was slightly decreased in cells transfected with $_{mut}XRE_{-1016/nqo1}$-Luc compared with cells transfected with $P_{-1016/nqo1}$-Luc. As was in the case of $Nrf2^{+/+}$ MEF, when DBA2/O fibroblasts were transfected with $_{mut}ARE/_{mut}XRE_{-1016/nqo1}$-Luc, the basal level were a slightly higher than cells transfected with construct containing a mutation only in ARE or XRE.

RL-34

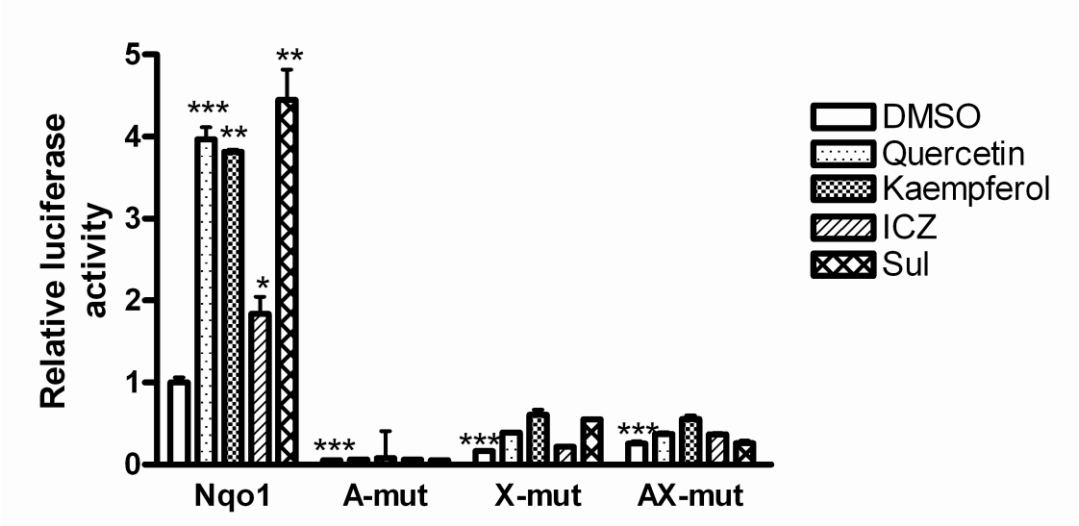


Figure 3.12 The ARE is responsible for both basal and inducible Nqo1-driven luciferase reporter activity.

RL-34 cells were seeded in 6-well dishes and left to recover for ~24 h to reach ~70% confluence before transfection was then carried out as described in Materials and Methods. RL-34 cells were co-transfected with 1.875 μg of each reporter for either $P_{-1016/nqo1}$-Luc (Nqo1), $ARE_{mut-1016/nqo1}$-Luc (ARE-mut), $XRE_{mut\ -1016/nqo1}$-Luc (XRE-mut) or $ARE_{mut}/XRE_{mut-1016/nqo1}$-Luc (AX-mut), together with 0.125 μg β-Gal plasmids for normalization. After transfection, cells were treated with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l), sulforaphane (5 μmol/l) or IC Z (1 μmol/l) for 24 h before luciferase activity was measured. Luciferase activity was normalized by β-Gal activity. (A) RL-34 cells. Values were presented as the fold induction by comparing all values with cells transfected with $P_{-1016/nqo1}$-Luc and treated with DMSO. Experiments were performed on at least three independent occasions. Data represents mean ± standard error. Student's t test was carried out for statistical analysis.（* P<0.01; ** P<0.05; ***P<0.001）.

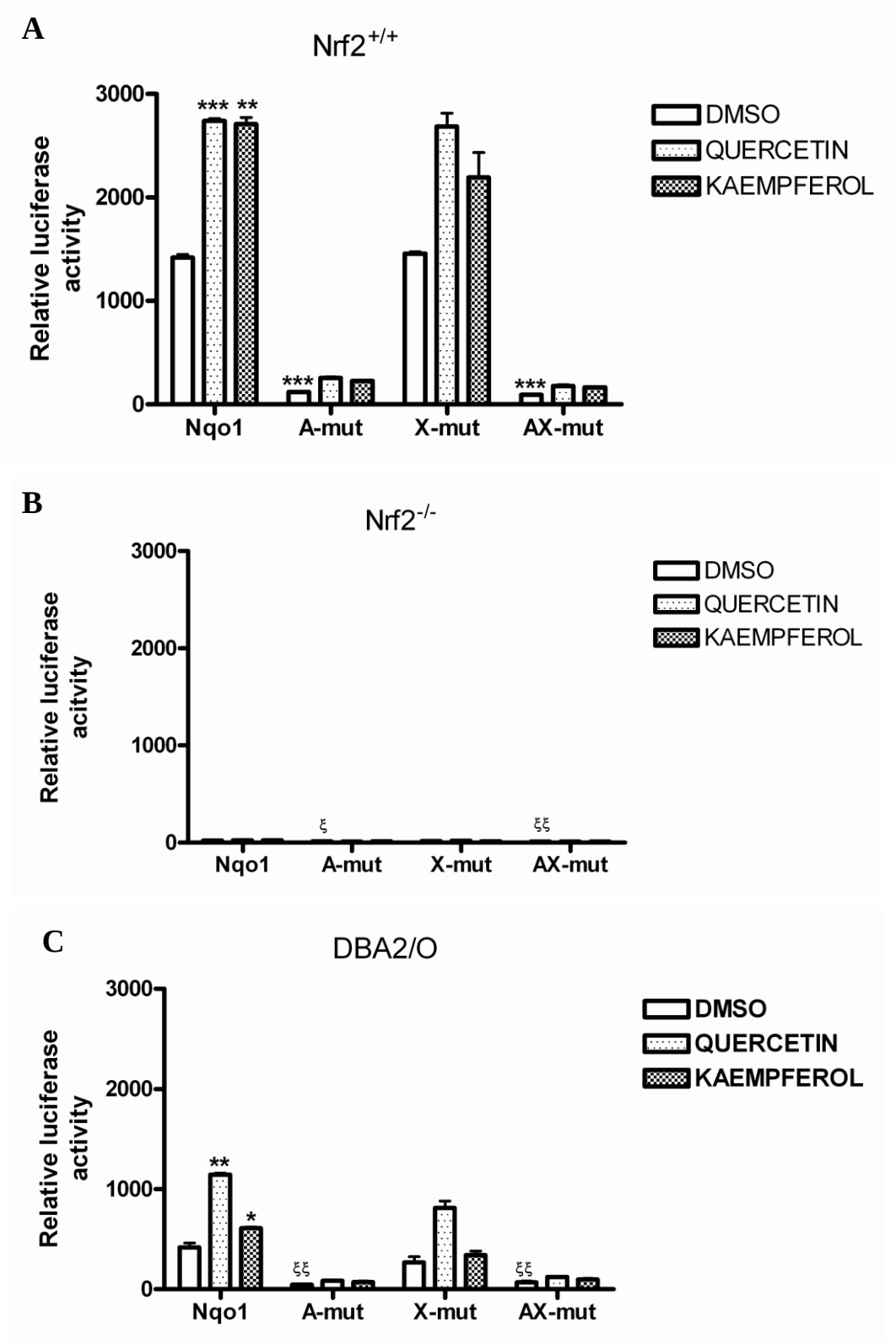


Figure 3.13 The ARE is responsible for both basal expression and induction of NQO1-driven luciferase activity.

Nrf2+/+, *Nrf2-/-* and BDAO/2 MEF cells were seeded in 6-well dishes and left to recover for ~24 to reach ~70%concluence. Transfection and treatment was carried out as described in Figure 3.13. Values were presented as the relative luciferase activity. Experiments were performed for on least

independent occasions. Data represent mean ± standard error. Student's t test was carried out for statistical analysis (* indicates a positive change while ξ indicates a negative change. */ ξ P<0.01; **/ ξ ξ P<0.05)

3.2.8 Generation of Deletion mutation constructs

When transversion mutations were introduced into both the ARE and XRE cis-elements, the basal level was higher than when either ARE or XRE was mutated alone. The reason for this finding is unclear. One possibility is that although the transversion mutation removed the ARE and XRE, it introduced another *cis*-element. To overcome this possible shortcoming, the ARE and XRE were eliminated by deletion mutagenesis. As described in Materials and Methods, the $P_{-1016/nqo1}$-Luc reporter construct was used as a template to generate three reporter constructs that lacked the ARE, the XRE or both of these elements, and were named as ΔARE $_{-1016/nqo1}$-Luc, ΔXRE$_{-1016/nqo1}$-Luc or ΔARE/XRE$_{-1016/nqo1}$-Luc, respectively. The deleted sequence for each construct is shown in Figure 3.14. Following transfection of RL-34 cells with the $P_{-1016/nqo1}$-Luc plasmid, treatment with quercetin and kaempferol induced luciferase activity between 3- and 4-fold (Figure 3.15). In MEF cells, quercetin and kaempferol also induced luciferase activity, though the fold increase was lower than that observed in RL-34 cells (Figure 3.16).

As shown in Figure 3.15 and 3.16 A, when cells were transfected with ΔARE $_{-1016/nqo1}$-Luc, both the basal and inducible reporter activities were dramatically decreased in RL-34 cells and also $Nrf2^{+/+}$ MEF cells. Interestingly, when Rl-34 cells

were transfected with ΔXRE $_{-1016/nqo1}$-Luc, the basal level of luciferase activity was decreased to about 50% of that obtained from cells transfected with the wild-type reporter plasmid (Figure 3.15). However, the magnitude of induction produced by quercetin and kaempferol from ΔXRE $_{-1016/nqo1}$-Luc did not change significantly when compared with the fold increase observed from cells transfected with the wild-type plasmid. Furthermore, in Nrf2$^{+/+}$ MEF cells, deletion of the XRE sequence increased basal luciferase activity and had no effect on induction (Figure 3.16A). When cells were transfected with ΔARE/ΔXRE$_{-1016/nqo1}$-Luc, basal and inducible luciferase activities were decreased to similar levels of that observed in the case of when cells were transfected with ΔARE$_{-1016/nqo1}$-Luc. Collectively, these results indicate that the ARE is indispensible for both basal and inducible expression of Nqo1 whereas the XRE may contribute to repression of the basal expression of Nqo1.

To investigate whether the changes in basal and inducible luciferase activities were dependent on Nrf2, we performed the same reporter gene experiment in *Nrf2*$^{-/-}$ MEF cells using wild-type and mutated P$_{-1016/nqo1}$-Luc as had been performed in *Nrf2*$^{+/+}$ fibroblasts. It is already known that Nqo1 is regulated by Nrf2 and our data showed consistently that the basal luciferase reporter activity was much lower in the mutant fibroblasts than in the WT MEF cells and that quercetin or kaempferol did not induce reporter gene activity in the mutant fibroblasts (Figure 3.16B).

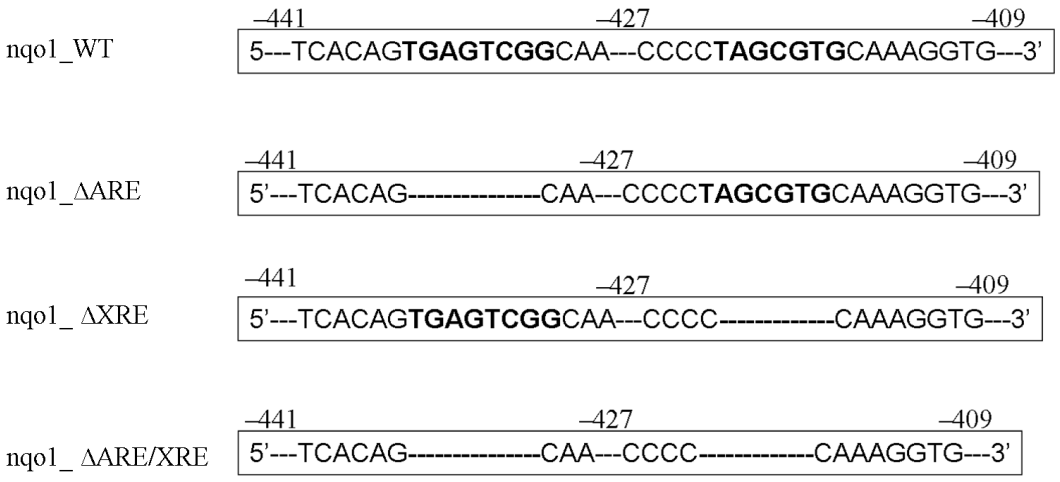


Figure 3.14 Sequences deleted in the mutant reporter constructs

Deletion mutagenesis was carried out as described in Materials and Methods. Bold letters in the wild-type construct indicate the sequence to be deleted in each of the mutant construct.

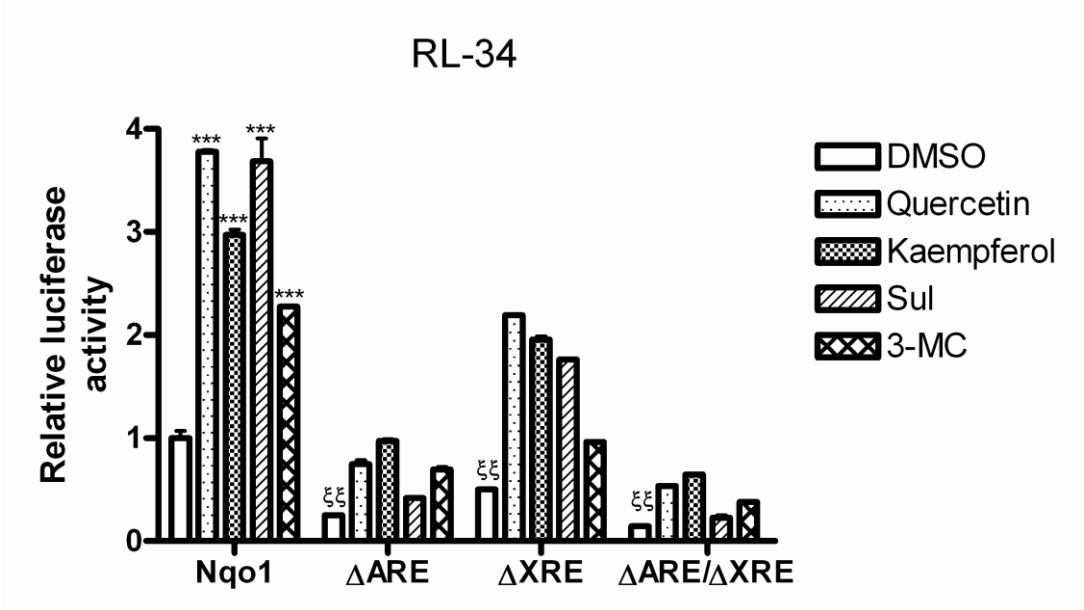


Figure 3.15 The ARE and XRE sequence exert different effects on induction of NQO1 by quercetin and kaempferol in RL-34 cells

RL-34 cells were seeded in 6-well dishes and left to recover for ~24 h to reach ~70% confluence before transfection was carried out as described in material and method. RL-34 cells were co-transfected with 1.875 μg of each reporter for either $P_{-1016/nqo1}$-Luc (Nqo1), ΔARE$p_{-1016/nqo1}$-Luc (ΔARE), ΔXRE $_{p-1016/nqo1}$-Luc (ΔXRE) or ΔARE/ΔXRE$p_{-1016/nqo1}$-Luc (ΔARE/ΔXRE), together with 0.125 μg β-Gal plasmids for normalization. After transfection, cells were treated with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l), sulforaphane (5 μmol/l) or 3-MC (1 μmol/l) for 24 h before luciferase activity was measured. Luciferase activity was normalized by β-Gal

activity. (A) RL-34 cells. Values are presented as the fold induction by comparing all values with cells transfected with $P_{-1016/nqo1}$-Luc and treated with DMSO. Data represent mean ± standard error. Student's t-test was carried out and p value was obtained by comparing all data set with cells transfected with $P_{-1016/nqo1}$-Luc and treated with DMSO. Student's t test was carried out for statistical analysis (* indicates a positive change while ξ indicates a negative change. */ξ P<0.01; **/ξξ P<0.05)

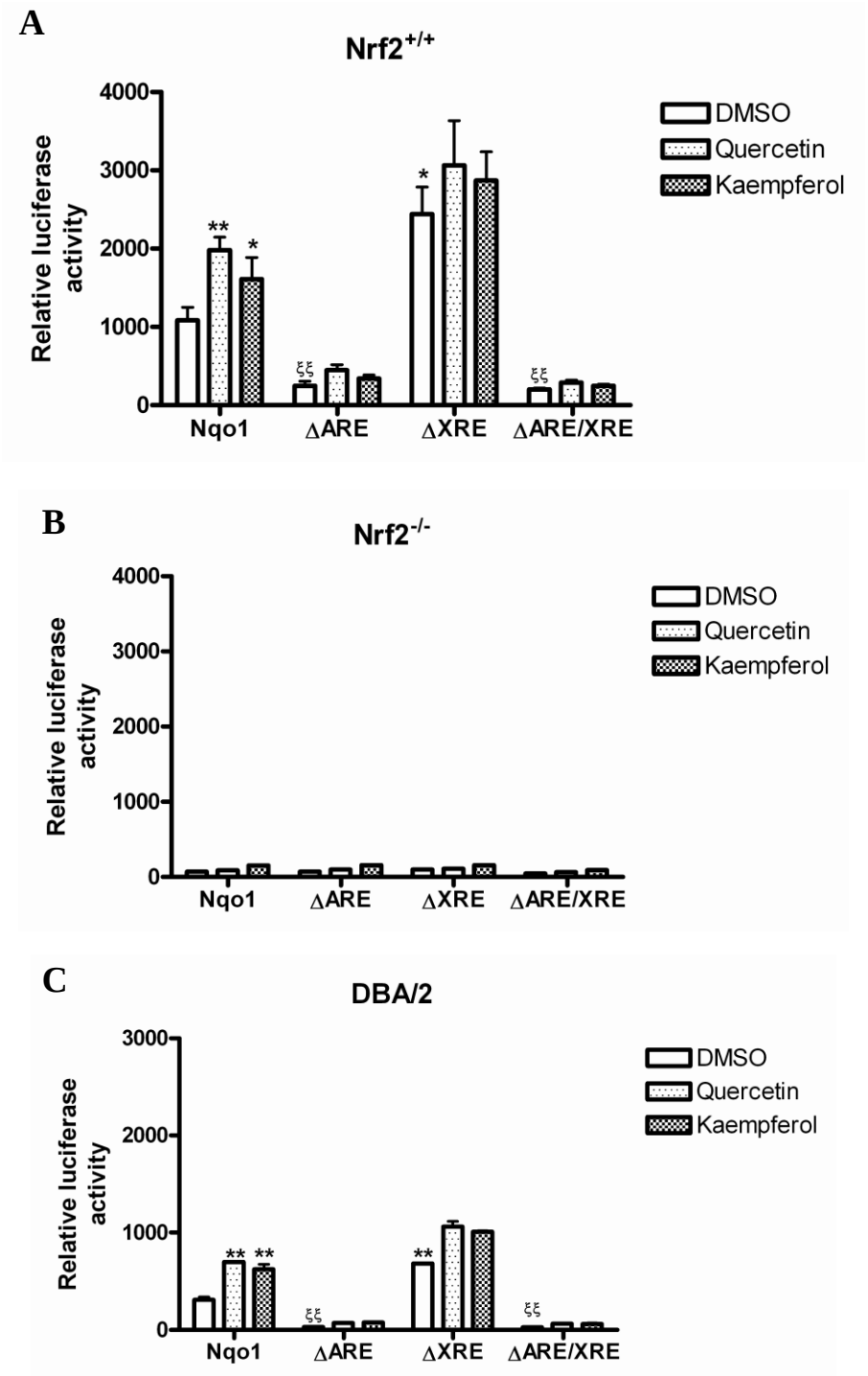


Figure 3.16 ARE and XRE exert different effects in the induction of NQO1 by quercetin and kaempferol in MEF cells

Nrf2+/+, *Nrf2-/-* and BDAO/2 MEF cells were seeded in 6-well dishes and left to recover for ~24 to reach ~70%concluence. Transfection and treatment was carried out as described in Figure 3.15. Values were presented as the relative luciferase activity. Experiments were performed for on least independent occasions. Data represent mean ± standard error. Student's t test was carried out for statistical analysis (* indicates a positive change while ξ indicates a negative change. */ ξ P<0.01; **/ ξ ξ P<0.05)

3.2.9 The effect of quercetin and kaempferol on the level of GSH

RL-34 cells were seeded in 60 mm dishes at a density of 10^6 cells per well, and left to recover for 24 h to reach ~90% confluence. Cells were then washed with PBS once and FBS-free medium was used to culture the cells for another 24 h. Chemicals were spiked into the medium at different time points and the cells were finally harvested simultaneously. Measurement of GSH was carried out as described in 2.4.9. The results in Figure 3.17 show that neither quercetin nor kaempferol affected the level of GSH significantly. By contrast, it was found that treatment with 5 μM Sulforaphane caused a depletion of intracellular GSH between 2-8 h of ~20 % and stimulated the synthesis of GSH causing a 1.5-fold increase.

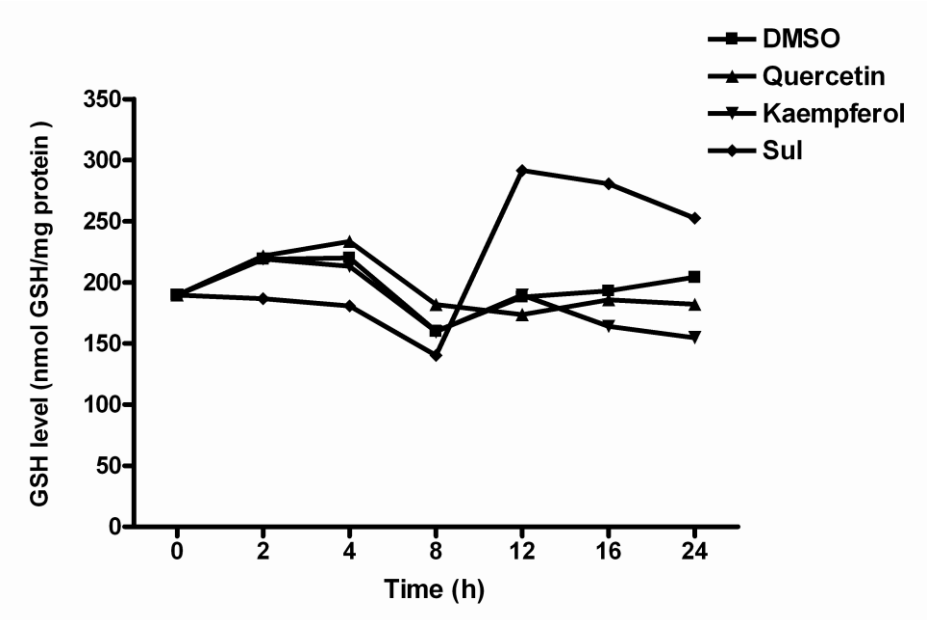


Figure 3.17 Changes in intracellular GSH upon treatment with phytochemicals

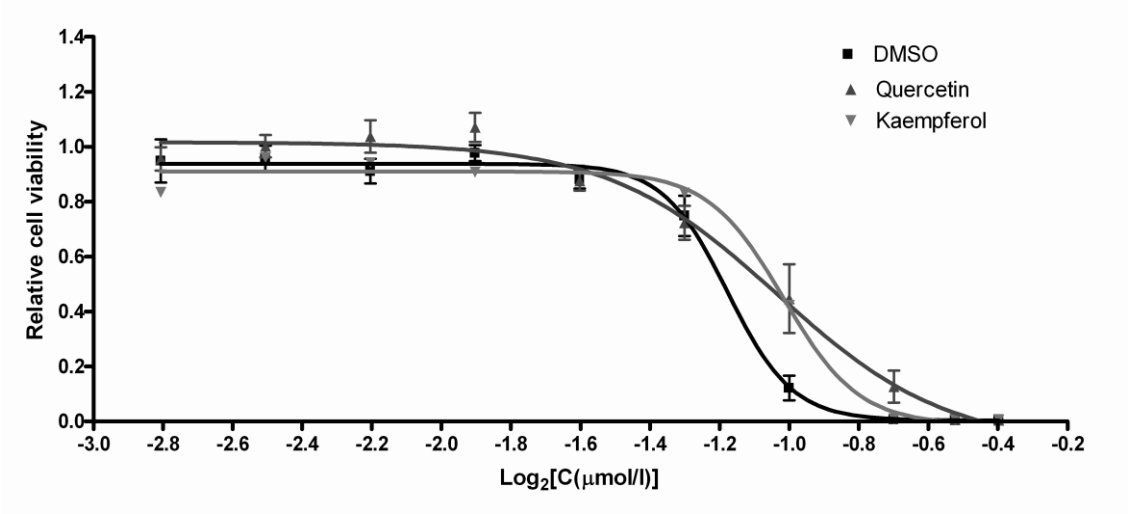
RL-34 cells were treated with quercetin or kaempferol at the final concentration of 20 μmol/l or sulforaphane at 5 μmol/l. The glutathione assay was carried out as described in Materials and Methods. For each sample, triplicate reaction was carried out. Total GSH was measured and normalized to the amount of protein and presented as nmol of GSH per mg of protein.

3.2.10 Biological consequence of treatment with quercetin and kaempferol

As our data have demonstrated that quercetin and kaempferol can induce ARE-driven gene expression, we tested the hypothesis that they could provide protection against electrophiles in an Nrf2-dependent fashion. For this purpose, we chose the reactive α,β-unsaturated aldehyde acrolein as the electrophile, the toxicity of which have been shown to be decreased by isothiocyanate sulforaphane (Higgins *et al.*, 2009). To this end, we pretreated RL-34 cells, *Nrf2*$^{+/+}$ MEF and *Nrf2*$^{-/-}$ MEF cells with a flavonoid for 24 h prior to challenge with acrolein for a further 24 hours. Cell viability was then measured by MTT. Statistical analysis was carried out by GraphPad Prism (GraphPad Software, 2236 Avendia La Playa La Jolla, CA 92037, USA). 95% confidence intervals are shown in the bracket following the EC_{50} values. It showed that in RL-34 cells treatment with quercetin or kaempferol can increase the EC_{50} from 66.5 (60.4-73.3) μmol/l to 92.5 (65.5-130.7) μmol/l or 96.2 (85.1-108.7) μmol/l respectively (Figure 3.18A). In MEF *Nrf2*$^{+/+}$ cells, EC_{50} was significantly increased by quercetin or kaempferol from 65 (63.5-66.6) μmol/l to 117 (106.9-128.6) μmol/l or 81 (75.8-85.8) μmol/l respectively (Figure 3.18 B). On the other hand, in *Nrf2*$^{-/-}$ MEF cells, the mutant fibroblasts treated with the vehicle had an EC_{50} of 16 (15.7-16.9) μmol/l (Figure 3.18 B), significantly lower than that of *Nrf2*$^{+/+}$ MEF. In

addition, the protection by quercetin and kaempferol was totally abolished. These results suggest that quercetin and kaempferol can provide protection against acrolein and this occurs in an Nrf2-dependent manner.

A RL-34



B MEF

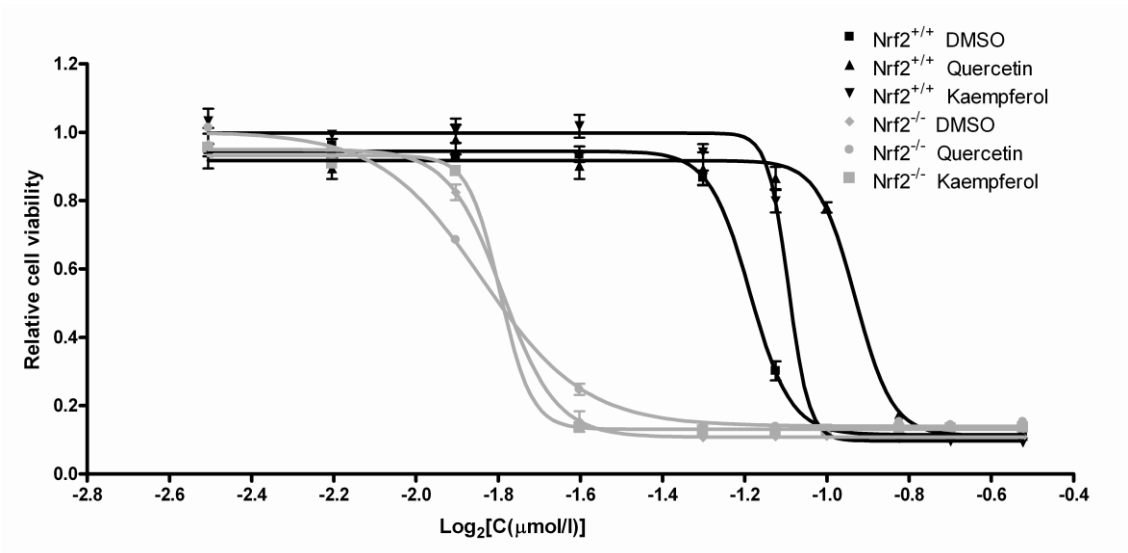


Figure 3.18 Quercetin and kaempferol protect against acrolein toxicity in an Nrf2-dependent manner.

RL-34 (A), *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ MEF cells were seeded in 96-well plates and left to recover for ~24 h to reach ~80% confluence. Cells were then pretreated with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l) or kaempferol (20 μmol/l) for 24 h. Afterwards, cells were treated for another 24 h with acrolein at various concentrations which are expressed as Log_2[C(μM)] in the figure. Experiments were performed on at least three independent occasions. Data represent the mean ± standard error.

Statistical analysis and student's t test were carried out and EC50 was calculated. (A) $P<0.05$; (B) $P<0.05$.

3.2.11 Induction of *Cyp1a1* mRNA by quercetin and kaempferol in RL-34 cells

The results shown above have demonstrated that the XRE in Nqo1 contributes to its basal expression and induction of Nqo1 by quercetin and kaempferol. This in turn suggests that quercetin or kaempferol may influence the expression of other XRE-driven genes, such as CYP1A1. To test this hypothesis, we examined whether quercetin and kaempferol increased mRNA level of *CYP1a1* in RL-34 cells. The result showed that both flavonoids could increase the mRNA level of CYP1A1 and the induction reached to a maximum at 6 h of 5.5-fold by quercetin and 3-fold by kaempferol (Figure 3.19).

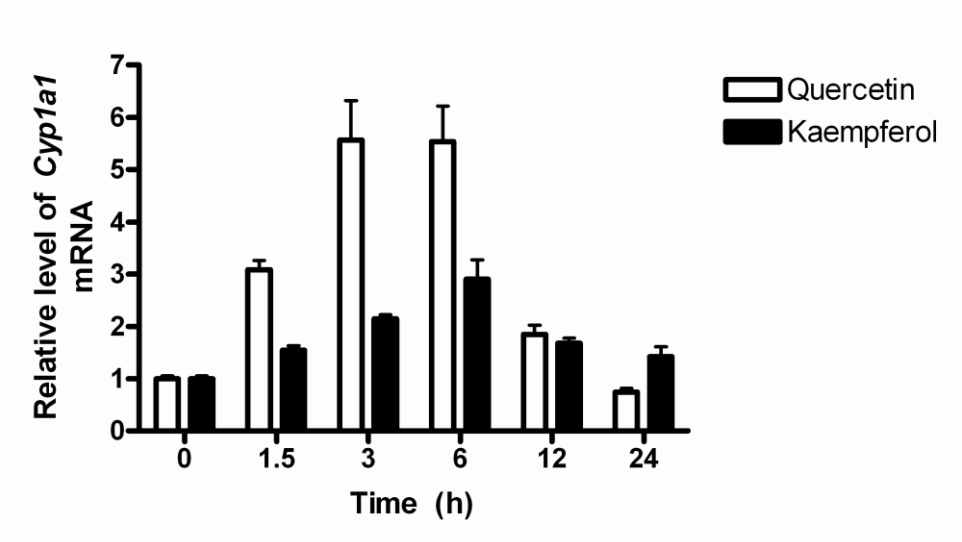


Figure 3.19 Quercetin and kaempferol can increase the mRNA level of CYP1A1
Experiment was performed in the same way in RL-34 cells as that described in Figure 3.10 and the level of CYP1A1 mRNA was measured by Taq-man and normalized to the mRNA level of actin. Experiments were performed for three separate occasions and the data is presented as mean ± standard error.

3.3 Discussion

3.3.1 Identificaiton of quercetin and kaempferol as inducers of ARE-driven genes

The ability of flavonoids from the sub-class of flavonol, flavanone, catechin tannin and anthocyan to induce ARE-drive genes was screened using AREc32 cells. The assay revealed that quercetin and kaempferol gave higher induction of ARE-driven luciferase activity than did other flavonoids. By contrast, their glycosides did not show comparative induction, indicating the aglycone form exerted more biological function. Narigenin from the flavonone family, caynidin, keracyanin chloride and oenin chloride from the anthocyan family give inductions less than 50%. For the catechin-tannins family, no induction was observed by any of the chemicals tested. The difference of the ability to induce ARE-driven gene presented between different flavonoids family is possibly structure-related. However, such a relationship needs further investment.

3.3.2 Induction of Nqo1 by flavonoids requires Nrf2

By Western blotting, it has been shown that both quercetin and kaempferol stimulate induction of Nqo1 protein. By contrast, they had no effect on the expression of GSTP1. Though the gene encoding GSTP1 contains a functional ARE sequence in its promoter region, it can be regulated in an Nrf2-independent pathway. These result suggested that the induction of ARE-driven gene by flavonoids is probably in an

Nrf2-dependent manner. To confirm the induction of Nqo1 by flavonoids is in an Nrf2-dependent manner, *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ fibroblasts were employed. This showed that compared with *Nrf2*$^{+/+}$ MEF, both the basal level and induction level of *Nqo1* by quercetin and kaempferol were substantially lower in *Nrf2*$^{-/-}$ MEF. Taken together, the induction of ARE-driven gene expression by flavonoids occurs through an Nrf2-dependent pathway.

3.3.3 Flavonoids did not alter the level of GSH

It has been reported that quercetin and kaempferol have antioxidant activity which is due to the presence of a phenolic hydroxyl group, and that quercetin exhibits a higher free radical scavenging activity than kaempferol (Akira *et al.*, 2008). In addition, studies on quercetin showed that during its antioxidative activities, it becomes oxidized into various products. The two electron oxidation of quercetin yields the oxidation product quercetin-quinone, denoted as QQ. An in-vitro study has shown that QQ is very reactive towards thiols and can instantaneously form an adduct with GSH and called GSQ, (Figure 3.18) (Galati *et al.*, 2001). This product is not stable and rapidly dissociates into GSH and QQ with a half life of 2 min. However, these studies are all *in vitro* and the *in vivo* formation and possible toxicity of QQ has not been demonstrated yet. In this theory, the possibility that quercetin and kaempferol can affect the level of endogenous GSH has been examined. This showed that the flavonoids exert no significant effect on the level of GSH. There are several possibilities that GSH was not depleted by either of the flavonoids. First, in the

absence of excessive [O] which was one of the conditions from previous studies examing the antioxidant property of quercetin and kaempferol, the flavonoids probably did not undergo oxidation, thus no reactive products were present to conjugate with GSH. Secondly, our study showed that the basal level of GSH is much higher in RL-34 cells than other cell lines (e.g. MEFs) (Higgins *et al.*, 2009) which may contribute to the low level of depletion of GSH by sulforaphane; even the flavonoids become oxidized in cells and conjugated to GSH, the change of GSH was not significant based on the high basal level. Such result suggested that the induction of NQO1 by quercetin and kaempferol is not due to their antioxidant property.

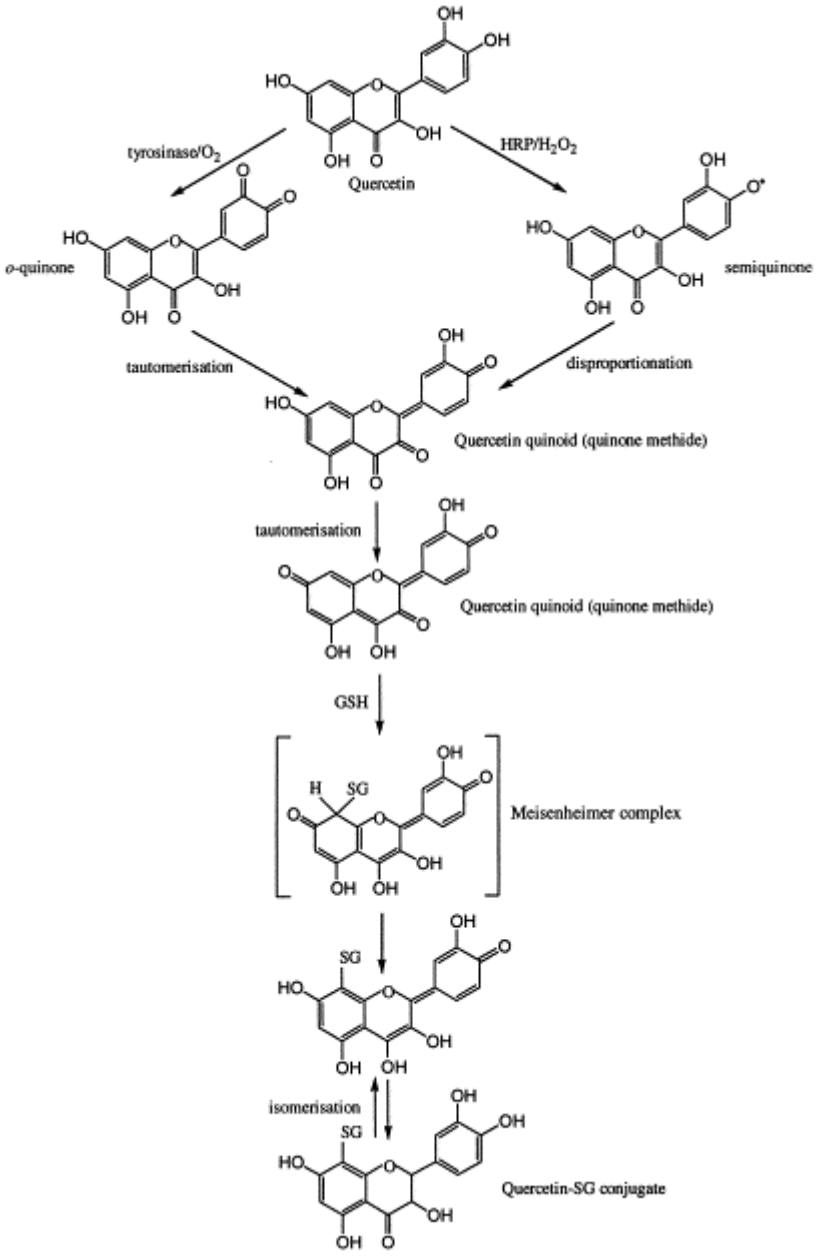


Figure 3.20 Schematic presentation of the peroxidative metabolism of quercetin.
The 3-hydroxy group on the C ring in quercetin can be oxidized to different products by the peroxidase-catalyzed one-electron oxidation. In the presence of GSH, quercetin forms a quercetin-SG conjugate with glutathione binding onto the A ring (Galati *et al.*, 2001).

3.3.4 The ARE but not the XRE is necessity for the induction of Nqo1 by flavonoids.

It was known that Nqo1 is regulated by Nrf2 through the ARE. However, recent

studies showed that Nqo1 can also be induced by the ligands of AhR (Ma *et al.*, 2004). In view of the facts that the promoter region of Nqo1 contains an XRE, and quercetin and kaempferol share structural similarities with typical AhR ligands, it seemed possible that these two flavonoids induce oxidoreductase through the AhR-XRE pathway. Our experiments have shown that these two flavonoids induce Nqo1 at both protein and mRNA level in an Nrf2-dependent manner. We have also examined whether Nrf2-dependent induction of Nqo1 requires a functional ARE and/or XRE. For this purpose, we carried out mutagenesis analyses. At first, transversion mutations were introduced to the core sequence of the ARE and/or XRE in the mouse *Nqo1* promoter. This showed that the ARE is required for induction of *Nqo1* gene expression. However, the introduction of transversion mutations into the XRE exerted different effects on basal Nqo1 expression and induction of Nqo1 by quercetin. In RL-34 cells, in the absence of a functional XRE, both the basal and inducible level of NQO1 decreased substantially though not to such a great degree as when the ARE was mutated. However, in *Nrf2*$^{+/+}$ MEF cells, it did not have significant effect on neither the basal nor induction level of Nqo1. Results from the DBA/2O MEFs also showed that the ARE but not XRE was required for basal and inducible expression of Nqo1 by flavonoids. Taken together, these results indicate that the ARE is necessity for both basal and inducible expression of Nqo1. However, there was a discrepancy in that when both ARE and XRE were mutated, in both RL-34 and MEF cells, the luciferase activity was higher than when only ARE was mutated.

We also examined the functional significance of the ARE and XRE sequence in the promoter of Nqo1 by deletion mutagenesis. Results from RL-34 cells and MEF cells showed that loss of the ARE abolished both basal and inducible expression of a reporter gene driven by the *Nqo1* promoter. Again, the absence of the functional XRE exerted distinct effects on *Nqo1*-driven luciferase activity in different cell lines. In RL-34 cells, it caused around 50% reduction in the basal and inducible level. By contrast, the absence of XRE stimulated an increase in NQO1-driven luciferase activity in both *Nrf2*$^{+/+}$ and DBA/2O MEFs. In the absence of both of these two response elements, the luciferase activity was lower than when either of them was deleted.

Collectively, the results in this chapter suggest that the ARE in Nqo1 is responsible for both its basal expression and its induction upon treatment with quercetin and kaempferol. As Nrf2$^{+/+}$ and DBA/2O MEFs are derived from two different mouse strains, the basal level of the luciferase activity is not comparable. The result from the DBA/2O fibroblasts suggests that lack of a functional AhR receptor did not affect the ability of quercetin or kaempferol to induce the *Nqo1*-driven luciferase activity; however, we could not draw the conclusion that AhR is not involved in the induction of NQO1 by the flavonoids, thus further investigation need to be carried out. The function of XRE for the induction of *Nqo1* is different for different cell lines. What caused such difference is still not clear.

3.4 Conclusion

The first part of my study showed that quercetin and kaempferol exhibit substantial ARE-inducing ability. They increased Nqo1 enzyme activity, induced Nqo1 protein levels, and stimulated the mRNA level of *Nqo1* in both RL-34 and MEF cells. Besides, the drug-metabolizing enzyme *Cyp1a1* was positively regulated by the flavonoid at the transcriptional level. Quercetin showed itself to be a better inducer of Nqo1 than kaempferol, which may be due to their different structures or their conversion to different metabolites in the cells. This point still needs further study.

Furthermore, it was found that induction of Nqo1 by the flavonoids occurs through an Nrf2-ARE dependent pathway. The XRE present in the promoter region of *Nqo1* appears to be involved in the induction of the oxidoreductase by the flavonoids in a cell-specific manner. Although previous studies showed that quercetin and kaempferol can act as pro-oxidants and be metabolized by conjugation with GSH, our study showed that in the cell culture system, the flavonoids did not alter the level of GSH. Finally, the induction of NQO1 by the flavonoids provides protection for cells against the toxicity cause by the electrophile acrolein.

4 Stimulation of Nrf2 and AhR activity by quercetin and kaempferol

4.1 Introduction

4.1.1 Nrf2

Nrf2 is a CNC-bZIP transcription factor that is responsible for the regulation of a battery of ARE-driven genes, mostly encoding drug-metabolizing enzymes and antioxidant enzymes such as glutathione S-transferases, glutamate-cysteine ligase, NQO1, UDP:glucoronosyl transferases (UGTs), heme-oxygenase-1 (HO-1) and peroxiredoxin-1 (Prx1) (Hayes & McMahon, 2001; Ishii *et al.*, 2000). These enzymes play a crucial role in protecting cells from electrophiles and oxidative stress, and prevent carcinogenesis. Under homeostatic conditions, Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), which facilitates degradation of Nrf2 through the proteasome (Itoh *et al.*, 2003). Some dietary flavonoids are thought to exert their beneficial effect by up-regulating ARE-driven genes in an Nrf2-dependent pathway.

More is known about the effect of quercetin on gene expression than is known about kaempferol. Tanigawa *et al.* (2007) investigated the mechanism by which quercetin increases the expression of NQO1 in human HepG2 cells. These workers found that

quercetin increased the binding activity of nuclear proteins to an ARE sequence and increased Nrf2-mediated gene transcription. The same study showed that quercetin increased the level of *NQO1* mRNA and the abundance of NRF2 protein, by inhibiting the ubiquitination and extending the half-life of Nrf2. Furthermore, quercetin also reduced the level of Keap1 protein through the modification of the BTB-Kelch protein, without affecting the association between Keap1 and Nrf2. A study by Kimura *et al.* (2009) showed that quercetin can increase NRF2 protein levels in human HaCaT keratinocytes and induce the expression of HO-1 and GCLM, thus preventing oxidative damage caused by UVA (Kimura *et al.*, 2009). A recent study of neuronal cells in culture has shown that quercetin is available intracellular just 1 min after treatment and it can increase the level of GSH around 40% but there is no effect on the protein level of thioredoxin (Trx2) (Arredondo *et al.*, 2010). In addition, these researchers showed that quercetin increased the mRNA level of GCLC and stimulated the nuclear translocation of Nrf2. Another research group has reported that treatment with quercetin can increase the expression of HO-1 and also its mRNA in rat basophilic leukemia 2H3 (RBL-2H3) cells and this was found to be associated with nuclear translocation of Nrf2 protein (Matsushima *et al.*, 2009). Also, Yao *et al.* (2009) have found that quercetin can protect human hepatocytes from ethanol damage by inducing HO-1. Moreover, this research group provided evidence that quercetin can modestly increase nuclear translocation of Nrf2 protein and this may be mediated by p38 and ERK (Yao *et al.*, 2007). Furthermore, investigation of age-related macular degeneration found that in human retinal pigment epithelial

APRE-19 cells, quercetin, and other flavonoids including fisetin and eriodictyol, could increase Nrf2 protein levels and induce expression of HO-1 and Nrf2 (Hanneken *et al.*, 2006).

Many studies have shown that kaempferol possesses antioxidant, anti-apoptosis and anti-inflammatory properties. In the case of kaempferol, though substantial studies have been reported for the broad effect of kaempferol on transcription factors such as NF-kB (Park *et al.*, 2009) and HIF-1 activity (Mylonis *et al.*, 2010) and also Src kinase activity (Lee *et al.*, 2010), only a small amount of studies were about its effect on Nrf2. To date, only one paper was found on Pubmed showing modulation of Nrf2 and its target gene by kaempferol. In this study, experiments carried out in House Ear Institute-Organ of Corti 1 HEI-OC1 cells showed that kaempferol prevented the ototoxic effects of cisplatin by stimulating nuclear accumulation of Nrf2 and inducing *HO-1* and *GCLC* mRNA (Gao *et al.*, 2010).

4.1.2 AhR

AhR is a ligand-activated transcription factor of the PAS superfamily. Ligand free AhR is located in the cytosol as a complex associated with Hsp90, p23 and XAP2. Ligand binding releases AhR from the complex and is followed by the translocation of AhR to the nucleus where it heterodimerizes with Arnt and binds to XRE sequences in the 5′ upstream region of target genes. Activation of AhR has been implicated in multiple physiological functions and dioxin-induced toxicology.

Ligands for the AhR, are structurally diverse. Many studies have investigated the role of flavonoids as the agonists/antagonists of AhR. Here, we will focus on studies describing such role for quercetin and kaempferol. In 1999, Ciolino *et al.* reported that quercetin served as an agonist of AhR whereas kaempferol acted as antagonist of AhR in MCF-7 cells (Ciolino *et al.*, 1999). In a later study in HepG2 cells, Ahsida *et al.* (2000) reported that quercetin and kaempferol exhibited antagonist activity for the nuclear translocation of AhR at a concentration range of 1-10 μmol/l, but no agonist activity was observed. (Ashida *et al.*, 2000). In 2003, Zhang et al. showed that quercetin and kaempferol were weak agonists of AhR-mediated gene expression in MCF-7 cells (Zhang *et al.*, 2003). The study by Zhang *et al.* also showed that the ability of quercetin and kaempferol to antagonize AhR was dependent on the cell context, as they did not exhibit such activity in HepG2 cells. In porcine vascular endothelial cells, quercetin inhibited induced oxidative stress produced by the PCB-77, induction of *CYP1A1* mRNA caused by PCB-77 and AhR-DNA binding stimulated by PCB-77 at concentration range of 10-100 μmol/l (Ramadass *et al.*, 2003). In Caco-2 cells, quercetin served as a weak inducer of CYP1A1 protein and mRNA; on the other hand, quercetin was found to be highly effective in suppressing CYP1A1 induction by 1nM TCDD (Pohl *et al.*, 2006). Kaempferol inhibited the formation of AhR/Arnt/DNA binding complex induced by β-NF and TCDD in vitro and in vivo, respectively (Puppala *et al.*, 2007). Using a cell-based reporter assay in HepG2 cells and human hepatocytes, Li *et al.* found that quercetin and kaempferol activate AhR (Li *et al.*, 2009). In human MCF-10A mammary epithelial cells,

quercetin increased *CYP1B1* and *CYP1A1* gene expression and activated AhR, whereas kaempferol suppressed constitutive CYP1B1 expression and antagonized AhR activation by benzo[α]pyrene (Rajaraman *et al.*, 2009). Taken together, these studies showed that quercetin and kaempferol can either serve as agonists or antagonists of AhR, possibly depending on the concentration of the flavonoids and cell context.

4.1.3 Possible interaction between Nrf2 and AhR

The AhR has been extensively studied for its role in the induction CYP1 enzymes and mediating the toxicity of dioxin-like chemicals. More recently, with the availability of AhR-null mice, it has been revealed that the receptor is involved in numerous physiological processes such as cell cycle, and embryonic development. The mechanisms underlying those functions are the cross talk between AhR and various signalling pathway. One of the signalling pathways is the Nrf2-ARE axis.

A few studies have investigated crosstalk between Nrf2 and AhR. Using Nrf2-null cells, Ma *et al.* (2004) showed that *Nrf2* in involved in the activation of NQO1 by TCDD, but the underlying mechanism was not established. Subsequently, Batist and his colleagues reported the Nrf2 mRNA in Hepa-1c1c7 cells was significantly induced by TCDD in an AhR-dependent way (Miao *et al.*, 2005). Further, these researchers also found that the promoter region of Nrf2 contains three XREs, and by chromatin immunoprecipitation assay, they showed AhR can bind to these XREs. In

another study, Kalthoff *et al.* (2010) showed that UGT1A10, one of the drug-metabolizing enzymes, can be regulated by both Nrf2 and AhR. The mRNA of UGT1A10 was showed to be induced by TCDD in KYSE70 human oesophageal squamous cell carcinoma cells. The promoter region of *UGT1A10* contains two functional XRE sequences and one ARE sequence, through which TCDD or tBHQ elicit induction of *UGT1A10*. In addition, the *UGT1A10* gene is coordinately regulated by Nrf2 and AhR. However, activation of AhR did not affect the mRNA level of Nrf2. The most interesting finding from this study was that Nrf2 and AhR can both bind the XRE and the ARE sequences, which explains the coordinate regulation of UGT1A10 by Nrf2 and AhR (Kalthoff *et al.*, 2010). A second possible mechanism is a direct interaction between AhR and Nrf2 proteins; however, no evidence has been presented so far to demonstrate that the two transcription factors can physically interact. A third possibility is that an interaction can occur between Nrf2 and AhR-associated proteins or an interaction between AhR and Nrf2-associated proteins.

4.1.4 Aims

Previous results using *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ MEF cells suggested that Nrf2 is required for the induction of *Nqo1* by quercetin and kaempferol. Overexpression of Nrf2 further increased the transactivation of *Nqo1* by phytochemicals.. We therefore wished to know whether these two flavonoids can increase Nrf2 activity. In addition, as quercetin and kaempferol can induce *Cyp1a1* mRNA, we wished to know whether

they could activate the AhR.

4.2 Results

4.2.1 Overexpression of Nrf2 or AhR can upregulate NQO1

Firstly, to confirm that Nrf2 and AhR are involved in the induction of NQO1 by quercetin or kaempferol, either or both of these two proteins were overexpressed in RL-34 cells that were co-transfected with $P_{-1016/nqo1}$-Luc. RL-34 cells were seeded in 6-well plates and allowed to reach ~80% confluence before they were transfected with various combinations of plasmid. After transfection, the cells were treated with phytochemicals for 24 h before luciferase activity was measured. A control group, comprising cells transfected with $P_{-1016/nqo1}$-Luc and the empty pcDNA3.1/A plasmid, demonstrated that the empty expression vector had no effect on luciferase activity. The results showed that forced expression of either Nrf2 or AhR elevated basal *Nqo1* luciferase expression as well as induction of reporter gene activity by quercetin kaempferol. When both of these transcription factors were co-transfected, the increase in Nqo1-luciferase expression was moderate and indicated that these two proteins had an additive effect on induction rather than a synergistic effect.

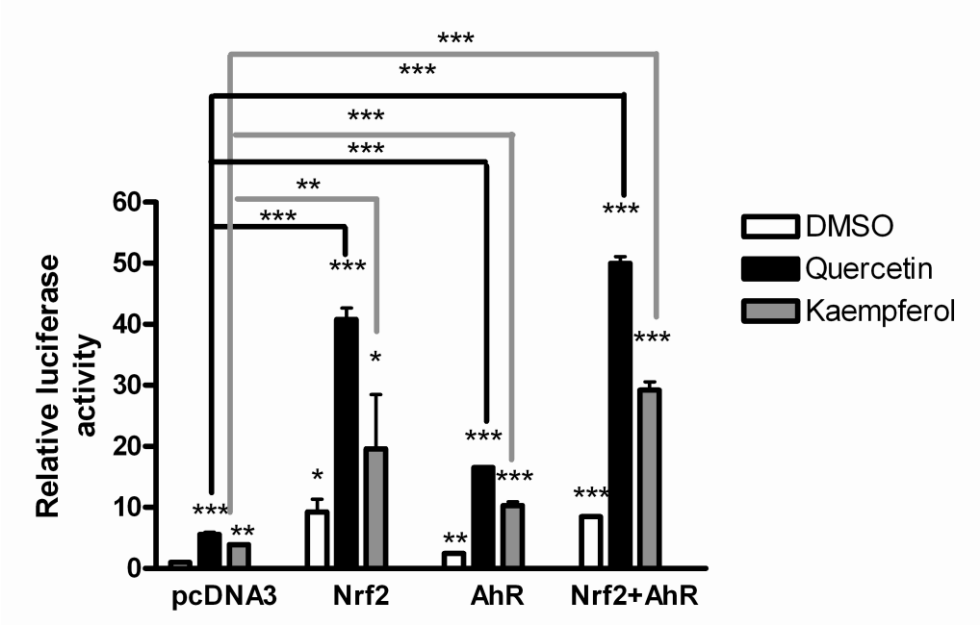


Figure 4.4 Enhanced induction of NQO1 luciferase activity upon over expression of Nrf2 and/or AhR.

RL-34 cells were seeded in 6-well plates. RL-34 Cells were co- transfected with 0.9375 µg $P_{-1016/nqo1}$-Luc along with 0.9375 µg empty pcDNA3 plasmid, Nrf2, AhR or both Nrf2 and AhR protein expressing vector and together with 0.125 µg β-Gal plasmids for normalization. After transfection, cells were allowed to recover for 16 h before they were treated with DMSO, quercetin or kaempferol. Experiments were carried out on at least 3 separate occasions. Global normalization was carried out for the average value of each occasions and data are presented here as the mean value plus ± standard error. All values were normalized to the value of samples which were transfected with $P_{-1016/nqo1}$-Luc and pcDNA3 and treated with DMSO. Student's t test was carried out by either comparing all the data sets with the DMSO group in the pcDNA3 data set, or comparing each treatment group in all data set with the same treatment group in the pcDNA3 data set. (. *P<0.01; ** P<0.05; *** P<0.001)

4.2.2 Nrf2 protein levels are increased by quercetin and kaempferol

Previous work has shown that under normal homeostatic conditions Nrf2 is negatively controlled by Keap1, which serves as a substrate adaptor for Cul3/Rbx1 and therefore targets the CNC-bZIP factor for ubiquitination. However, under stress conditions, Keap1 is inactivated and Nrf2 protein is stabilized resulting in induction of ARE-driven genes. To check whether quercetin and kaempferol induce NQO1 expression by stabilizing Nrf2 protein, endogenous levels of the CNC-bZIP factor in

RL-34 and MEF cells were examined after they had been treated with polyphenols for 2 h. Sulforaphane and 3-MC were included as positive controls. Immunoblotting showed that in both cell lines, the endogenous level of Nrf2 protein was increased by the polyphenols. In RL-34 cells, 20 μM quercetin and 20 μM kaempferol increased Nrf2 protein levels by ~9-fold and 5-fold, respectively; by comparison, sulforaphane (5 μmol/l) elicited an increase of ~ 9-fold as well, but 3-MC, a typical ligand of the AhR had no effect on the amount of Nrf2 protein. In *Nrf2*$^{+/+}$ MEF cells, all xenobiotics tested had a more modest effect on Nrf2 protein compared with their effects in RL-34 cells. Quercetin and kaempferol caused ~3.5-fold and 3-fold increases in Nrf2 protein, respectively; sulforaphane gave ~3-fold increase whereas 3-MC decreased the level of Nrf2 protein in MEFs to 50% compared with that of fibroblasts treated with DMSO.

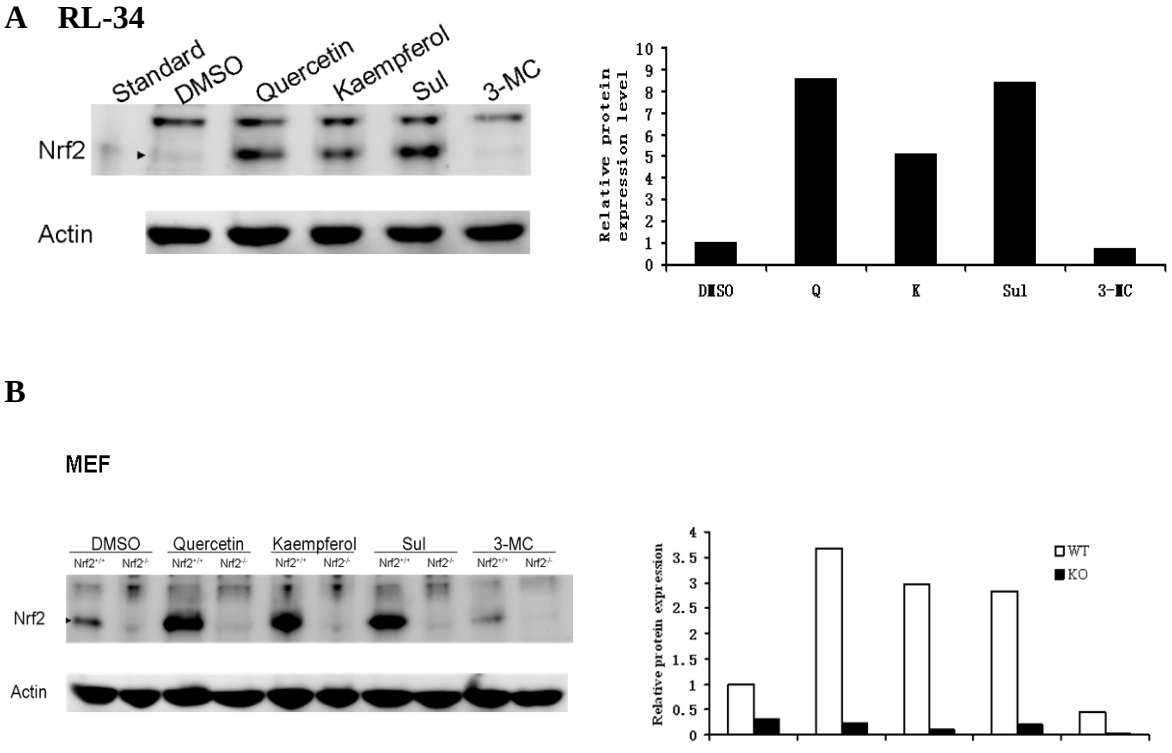


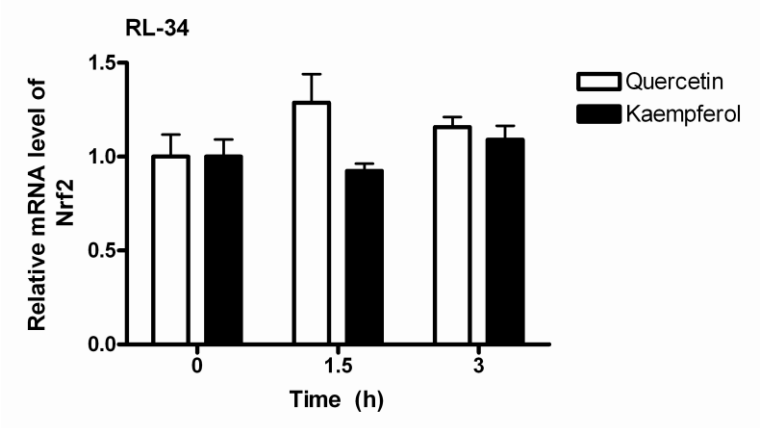
Figure 4.5 The level of Nrf2 protein is increased by quercetin and kaempferol

(A) RL-34 cell were treated with vehicle DMSO (0.1 %, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l), sulforaphane (5 μmol/l) and 3-MC (1μmol/l) for 2 h. Nrf2 protein level was monitored by Western blotting (left panel). Densitometry analysis of the immunoblot using quantity one software is presented in the right-hand panel. Analysis was performed by comparing treated cell with untreated cells. Density of the band corresponding to Nrf2 was normalized to that of actin before analysis. (B) $Nrf2^{+/+}$ and $Nrf2^{-/-}$ MEF cells were exposed to same treatment as that for RL-34 cells for 2 h and Nrf2 protein level was monitored by Western blotting (left panel). Densitometry analysis of the immunoblot was presented on the right-hand panel. Analysis was performed by comparing treated $Nrf2^{+/+}$ MEF cells and both treated and untreated $Nrf2^{-/-}$ MEF cells with untreated $Nrf2^{+/+}$ MEF cells.

4.2.3 Transcription of *Nrf2* is not affected by quercetin and kaempferol

Having shown that the level of Nrf2 protein can be increased by quercetin and kaempferol, we examined whether the elevation was due to increased transcription of the *Nrf2* gene. As the increase in Nrf2 protein occurred within 2 h, we treated cells with quercetin or kaempferol for either 1.5 or 3 h. Cells were then harvested and total mRNA was extracted and *Nrf2* mRNA was quantified as described previously. This showed that the mRNA for Nrf2 did not change significantly over these time points in either RL-34 or MEF cells. This result indicated that the increased abundance of Nrf2 protein in RL-34 and MEF cells was not due to the increased transcription of *Nrf2*.

A



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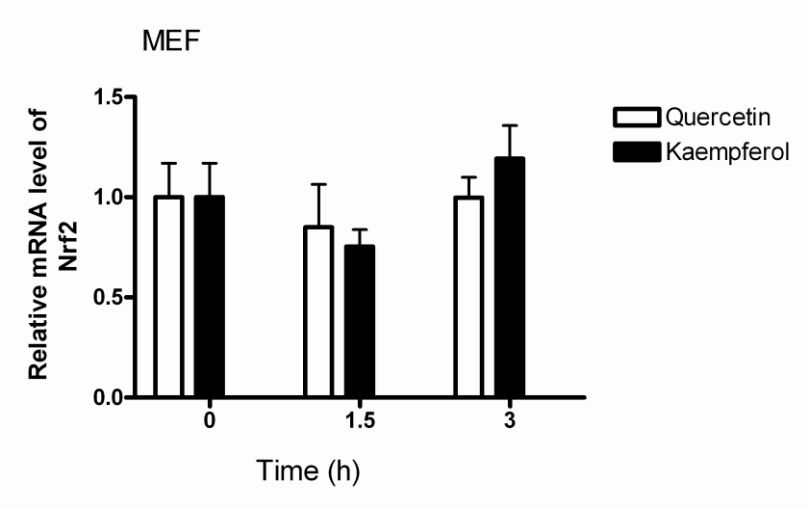


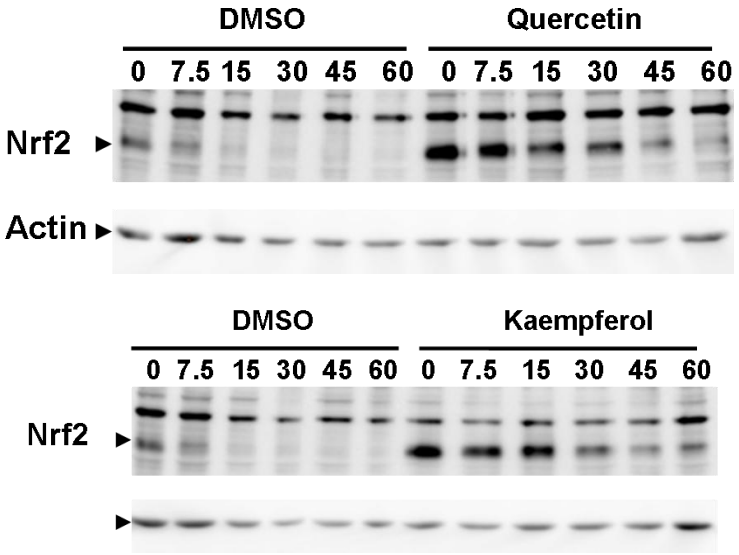
Figure 4.6 The mRNA level of Nrf2 is not regulated by quercetin or kaempferol
RL-34 (A) or MEF (B) cells were treated with vehicle DMSO (0.1%) or quercetin (20 μmol/l) or kaempferol (20 μmol/l) for either 1.5 or 3 h. Taq-man was performed as described in Materials and Methods. CT value of Nrf2 was normalized to that of actin. Values represent the mean ± S.E. of three independent measurements.

4.2.4 Stabilization of Nrf2 protein by quercetin and kaempferol

4.2.4.1 The half-life of Nrf2 protein is increased by quercetin and kaempferol

Nrf2 can be regulated through post-translational mechanisms; we were interested to know whether quercetin or kaempferol could stabilize the CNC-bZIP protein. For this purpose, a cycloheximide-chase experiment was carried out as described in section 2.4.5. The data showed that the half-life of Nrf2 protein in RL-34 cells pre-treated with DMSO was around 30 min while it increased to 56 and 60 min in cells pre-treated with quercetin or kaempferol, respectively.

A



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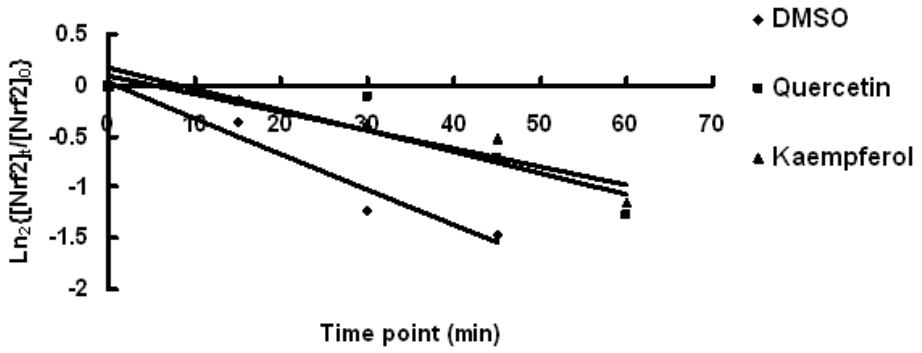


Figure 4.7 The half-life of Nrf2 protein is increased by quercetin and kaempferol

(A) RL-34 cells were treated for 2h with DMSO (0.1%, v/v), quercetin (20 μmol/l), or kaempferol (20 μmol/l) before CHX (10 μmol/l) were spiked into the medium at different time points. Cells were then harvested simultaneously with exposure to the chemicals for various time intervals. Nrf2 protein was detected by Western blotting. (B) Densitometry analysis was carried out using Quantity one software and the calculation of half-life of Nrf2 protein is described in Materials and Methods.

4.2.4.2 Effect on the ubiquitylation of Nrf2 by quercetin and kaempferol

The cycloheximide-chase experiment showed that quercetin and kaempferol both stabilize the Nrf2 protein in RL-34 cells. However, it is not clear through what mechanisms such stabilization occurred. McMahon, *et al.* (2003) has shown that Nrf2 is subject to ubiquitination in a Keap1-dependent manner. Thus one hypothesis

was that the half-life of Nrf2 protein increased as a consequence of its Keap1-mediated ubiquitination being inhibited. To test this idea, we examined the stability of ectopic Nrf2 when expressed in COS-1 cells in the presence or absence of ectopic mKeap1. To normalize the transfection efficiency, an expression construct for β-Gal was cotransfected with plasmids for mNrf2-V5 and mKeap1. After transfection, the COS-1 cells were treated with various phytochemicals for 2 h to see whether they could stabilize mNrf2-V5 protein in the presence of mKeap1. As anticipated, the level of ectopic Nrf2 protein in COS-1 cells that had been co-transfected with mKeap1 was much lower than when mKeap1 was absent. When cells were transfected with both mNrf2-V5 and mKeap1 expression plasmids, quercetin and kaempferol increased the level of Nrf2 ~4-fold and 2-fold, respectively.

After showing that quercetin and kaempferol could stabilize Nrf2 protein, we carried out the ubiquitin assay as described in 2.4.7. The COS-1 cells were transfected with various combinations of mNrf2-V5, mKeap1 and Ub-his. After transfection, cells were treated for 2 h with various phytochemicals. Subsequently, His-tagged proteins were affinity-purified and subjected to Western blotting. A smear of high molecular mass V5-tagged protein was detected in the “pull-down” fraction contransfected with mNrf2-V5 and Ub-His (Figure 4.5 lanes 1-5) but not when either mNrf2-V5 or Ub-His was present. This result suggested that the high molecular weight fraction in the smeared band represents the polyubiquitinated mNrf2-V5. Densitometry analysis was carried out and the ratio of ubiquitinated Nrf2 protein to the amount of total Nrf2

protein was calculated. All the values were divided by the ratio when cells were treated with DMSO and the result obtained is presented as the fold inhibition of ubiquitination of Nrf2 protein. This experiment showed that both quercetin and kaempferol can inhibit the ubiquitination of Nrf2 by 50%.

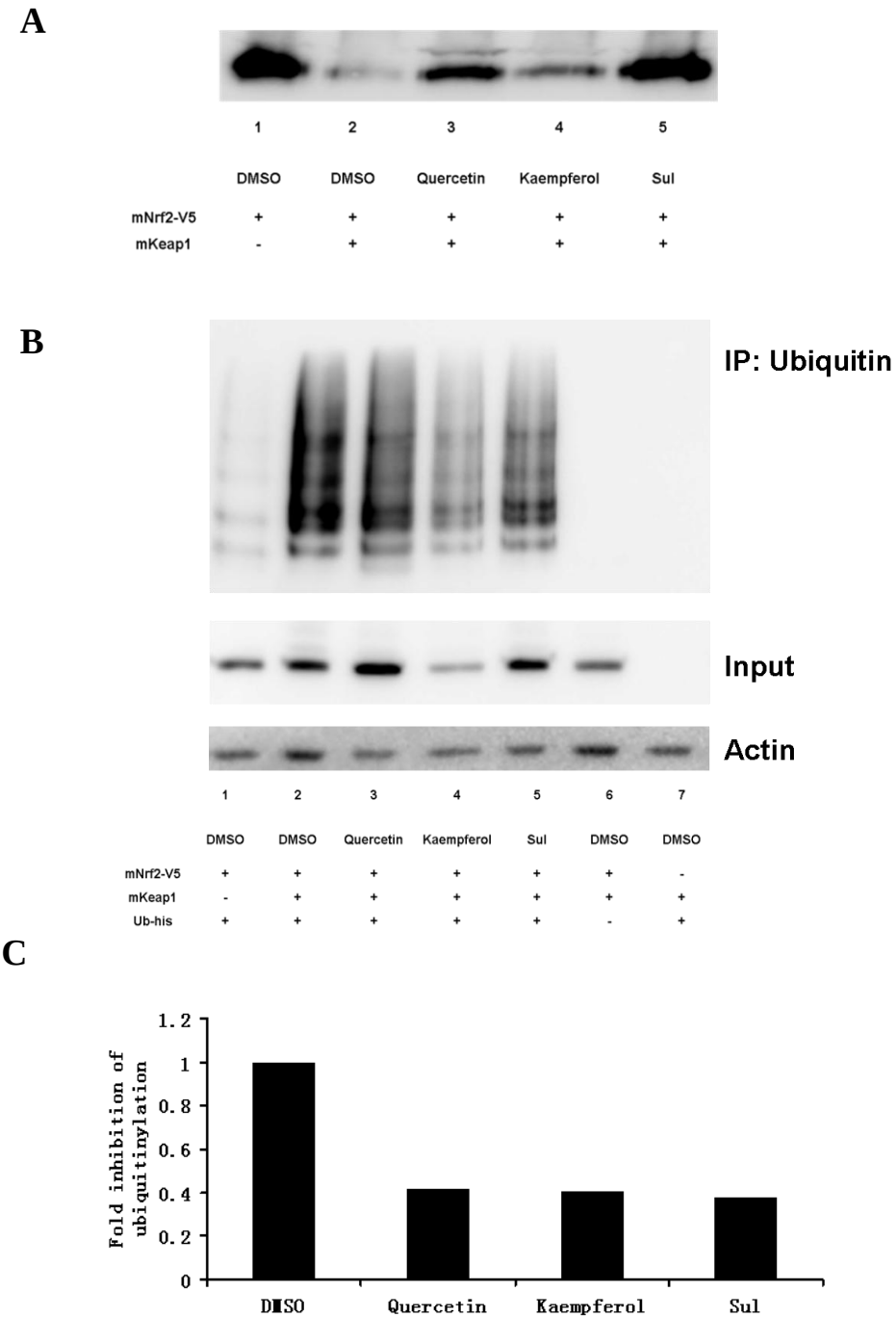


Figure 4.8 Quercetin and kaempferol can inhibit the ubiquitination of Nrf2 protein.
(A) COS-1 cells were transfected with mNrf2-V5 either in the presence or absence of mKeap1. After transfection, cells were treated for 2 h with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), kaempferol (20 μmol/l) or sulforaphane (5 μmol/l). The amount of protein used for analysis of each sample was normalized by the activity of β-Gal.
(B) COS-1 cells were transfected with expression constructs for mNrf2-V5 and Ub-his in the presence (lane 2-5) or absence of mKeap1 (lane 1). In the presence of mKeap1, cells transfected with only mNrf2-V5 (lane 6) or Ub-his (lane 7) were included as negative control to prove the smear high molecular weight band represent ubiquitinated Nrf2 protein was measured. After transfection, the cells were treated as described in (A) for 2 h before the ubiquitination assay was carried out as described in Materials and Methods. The amount of V5-tagged protein in the samples from both "IP" and "input" was measured using Western blotting. For the input blot, it was stripped and re-probed with anti-actin antibody.
(C) Densitometry analysis was carried out using quantity one software. The ratio of the band density of the ubiquitinated Nrf2 to that of the corresponding intact Nrf2 protein was calculated. The ratio of treated cell was compared with the untreated cells.

4.2.5 Quercetin and kaempferol change the cellular localization of Nrf2

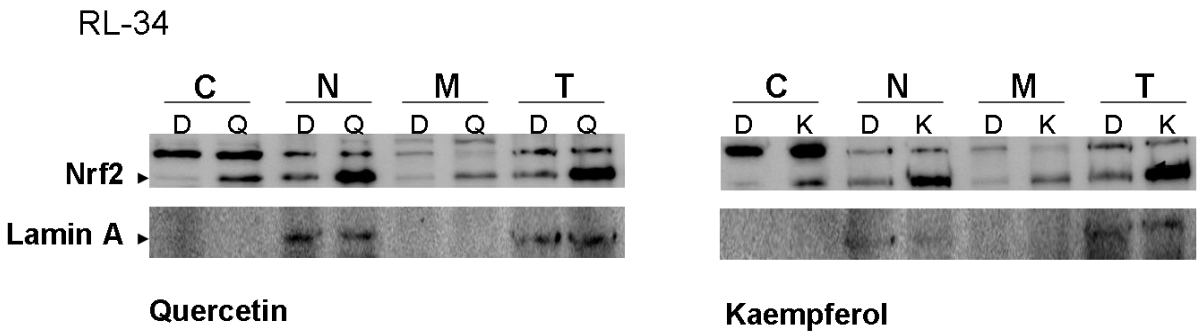
The cyclohexamide-chase experiment showed that quercetin and kaempferol can stabilize Nrf2 protein. As we wished to know where such stabilization occurred, we examined whether the phytochemicals change the subcellular distribution of Nrf2 protein. Cellular fractionation and immunocytochemistry were employed to address this question.

4.2.6 Cellular fractionation

RL-34 and MEF cells were treated for 2 h with various chemicals before being harvested and subjected to subcellular fractionation as described in 2.3.5. Immediately prior to fractionation, a small portion of total lysate (T) was withdrawn for immunoblot analyses along with protein from different subcellular fractions. In the case of RL-34 cells, three fractionations were purified (i.e. cytosol (C),

membranes (M) and nuclei (N)), whereas in the case of MEF cells, only two fractions were purified (i.e. the 3000× *g* (S) supernatant, containing both cytosol and membranes, and the nuclei (N)). Lamin A was used as a control to allow the purity of each fraction to be monitored. Western blotting showed that in both cell lines, quercetin and kaempferol increased the amount of Nrf2 protein in both the cytosol (i.e. 3000× *g* fraction in MEF cells) and the nuclear fractions. However, the blots suggested Nrf2 protein was present predominantly in the nuclear fraction rather than the cytoplasm under both basal and stressed conditions.

A



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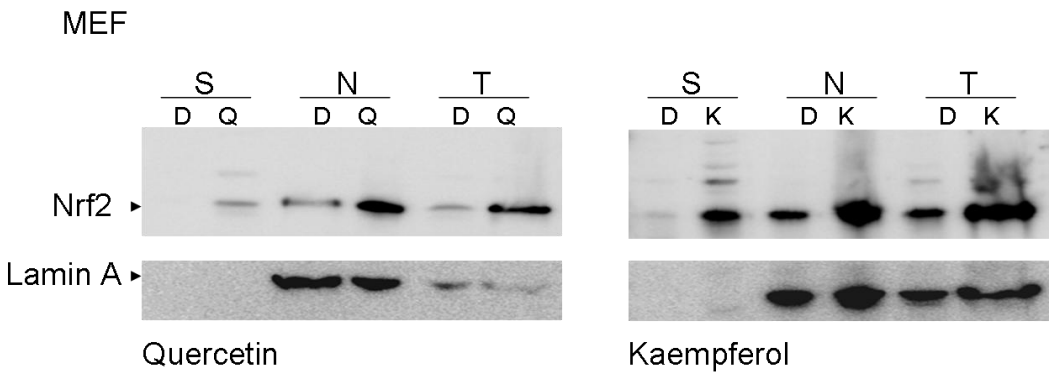


Figure 4.9 Nrf2 protein is present predominantly in the nucleus both under basal and stressed condition.

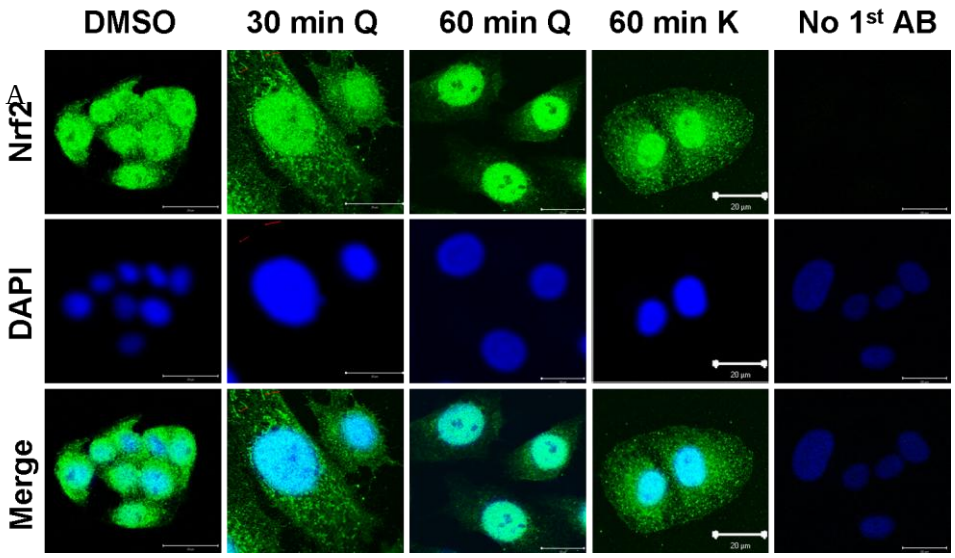
RL-34 (A) and Nrf2 $^{+/+}$ MEF cells (B) were treated for 2 h with vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l), or kaempferol (20 μmol/l), after which time the cells were subjected to subcellular fractionation as described in Materials and Methods. For RL-34 cells, three fractions were isolated

including cytosol (C), nucleus (N) and membrane (M). For MEF cells, two fractions were isolated including a 3000 × *g* fraction which included both cytosol and membranes (S) and nucleus (N). Total lysates (T) were also loaded as a positive control along with other fractions. The level of Nrf2 protein from each fraction was monitored by Western blotting. The same blot was stripped and re-probed with lamin A to evaluate the purity of nuclear fractions.

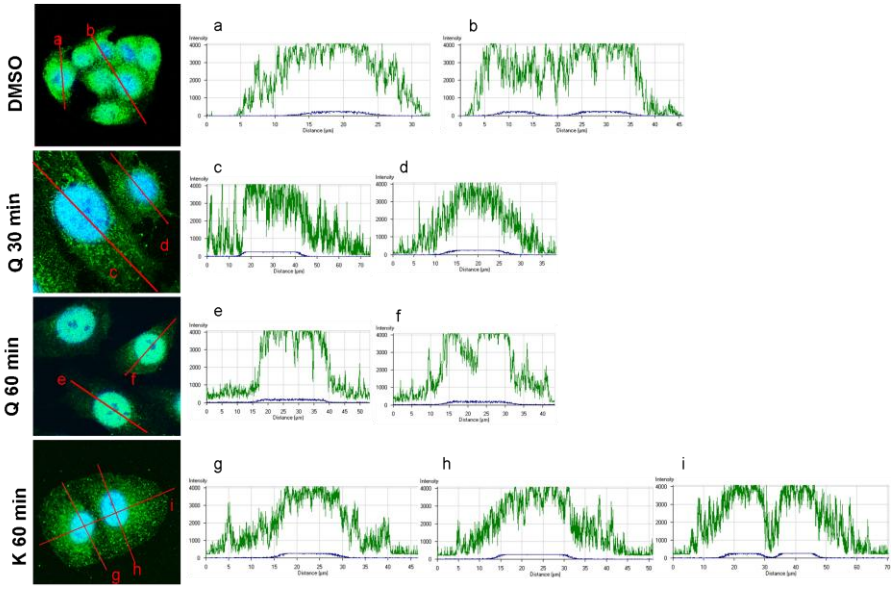
4.2.6.1 Immunocytochemistry

To further investigate the effect of quercetin and kaempferol on the cellular localization of Nrf2 protein, RL-34 cells were treated with each flavonoid for either 30 min or 60 min before immunocytochemistry was carried out as described in 2.3.4. Consistent with the subcellular fractionation data, immunocytochemistry revealed that in both the untreated cells and those that had been treated with flavonoid, Nrf2 was observed predominantly in the nucleus. However, upon treatment with quercetin or kaempferol, the amount of Nrf2 protein present in the nucleus, relative to that in the cytoplasm, was increased at both the 30 and 60 min time points. Thus these results also indicate that quercetin and kaempferol can stabilize Nrf2 protein and increase its abundance in the nucleus. Furthermore, it was noteworthy that in cells which were not exposed to xenobiotics, about 5% of them had more Nrf2 in cytoplasm than in the nucleus. The reason for this requires further investigation.

A


DMSO
30 min Q
60 min Q
60 min K
No 1st AB
Nrf2
DAPI
Merge
20 µm

B


DMSO
Q 30 min
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C

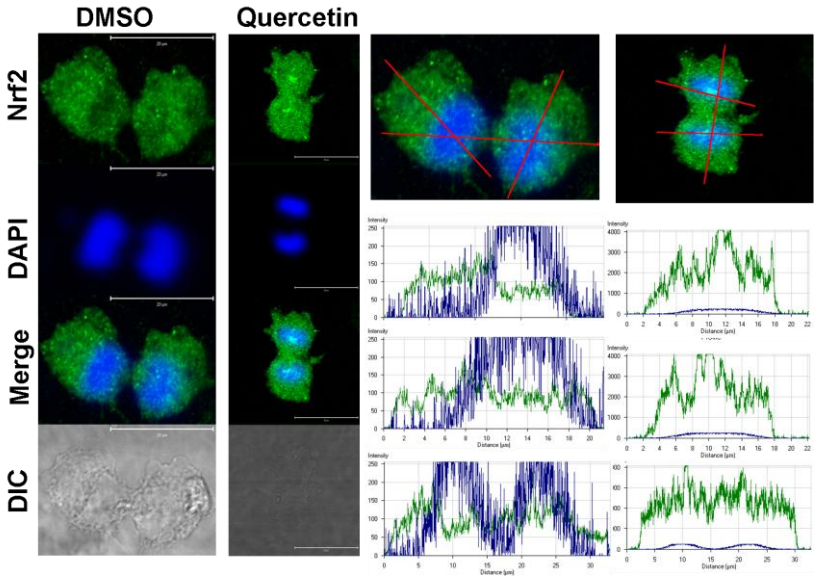


Figure 4.10 Nrf2 protein accumulates in the nucleus upon treatment with polyphenols.

RL-34 cells were seeded onto coverslips in 60-mm dish and allowed to recovered for ~16 h by which time they had reached ~40-60% confluence. Afterwards, the cells were treated with vehicle DMSO (0.1%, v/v), quercetin (20 µmol/l) (A, B and C)) and kaempferol (20 µmol/l) (C & D) for various periods of time. As stated in Materials and Methods, location of endogenous Nrf2 protein was examined by immunocytochemistry followed by confocal imaging. FITC-labelled secondary antibody was used to locate Nrf2 protein. Nuclear DNA was stained by DAPI. DIC indicates images from normal light microscopy. The merge signal reprsents the results obtained when the three images were superimposed.

(A) Confocal images of the localization of Nrf2 proteins. (B) The profiling of the distribution of the Nrf2 protein. (C) 5% of cell having Nrf2 predominantly accumulated in the cytoplasm only when treated with DMSO.

4.2.7 AhR protein level was not affected by quercetin

Due to the low affinity of the AhR antibody, it was not possible to detect signal by Western blotting. Therefore, immunoprecipitation was carried out as described earlier.

To confirm the authenticity of the band believed to correspond to AhR, the mRNA for the receptor was also knocked down using siRNA. Prior to immunoblotting, cells were transfected with AhR siRNA or scrambled siRNA, and allowed to recover for

16 h before being subjected to immunoprecipitation. A comparison was made between cells transfected with AhR siRNA and those transfected with scrambled siRNA; and comparing cells pulled down with goat anti AhR antibody and cells pulled down with anti-goat IgG, the band indicated by an arrow corresponded to the AhR protein (Figure 4.8). A time course experiment was carried out for the immunoprecipitation and this method provided no evidence that the level of AhR protein was changed by quercetin

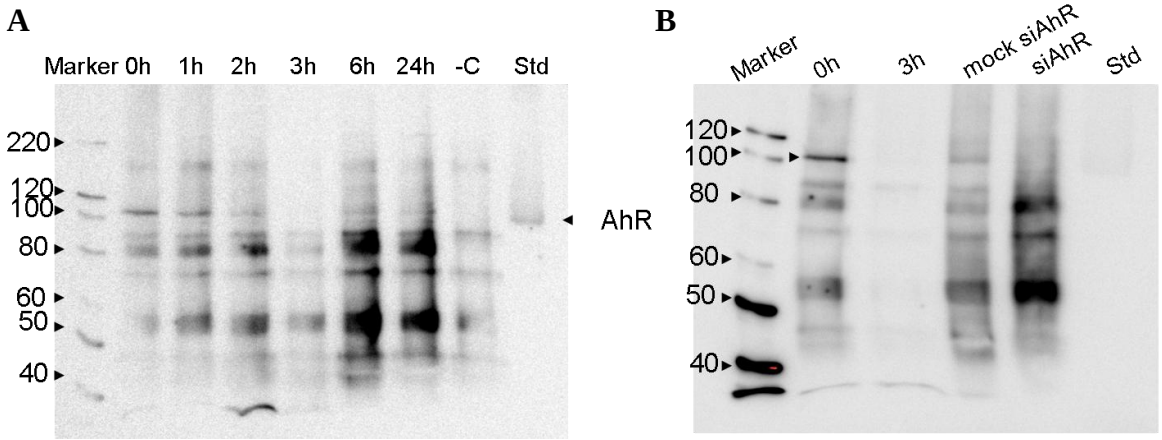


Figure 4.11 Immunoprecipitation suggested there was no change of the level of AhR protein.
(A) Two 100 mm dishes of RL-34 cells were seeded and left to reach ~80% confluence. They were then treated with vehicle DMSO (0.1%, v/v) or quercetin (20 μmol/l) for various times, as indicated, and harvested simultaneously. Immunoprecipitation was then carried out as described in Materials and Methods.
(B) "Mock siAhR" or siAhR represents samples obtained from cells that were first transfected with either mock siRNA or AhR siRNA, respectively. After transfection, they were allowed to recover for 24 h before they were subjected for immunoprecipitation as stated in (A).
Arrow in (A) indicated the AhR standard in (B) indicated the actual band corresponding to AhR protein. "-C" represent the sample that were not pulled down by anti-AhR antibody but goat IgG.

4.2.8 Quercetin and kaempferol up-regulate the AhR mRNA level

RL-34 cells were treated with quercetin or kaempferol for different periods before

being harvested at the same time. RNA was extracted and measured as described earlier. This showed that quercetin and kaempferol gave the highest increase of 3.5-fold and 1.8-fold in the amount of *AhR* mRNA , respectively, at 12 h.

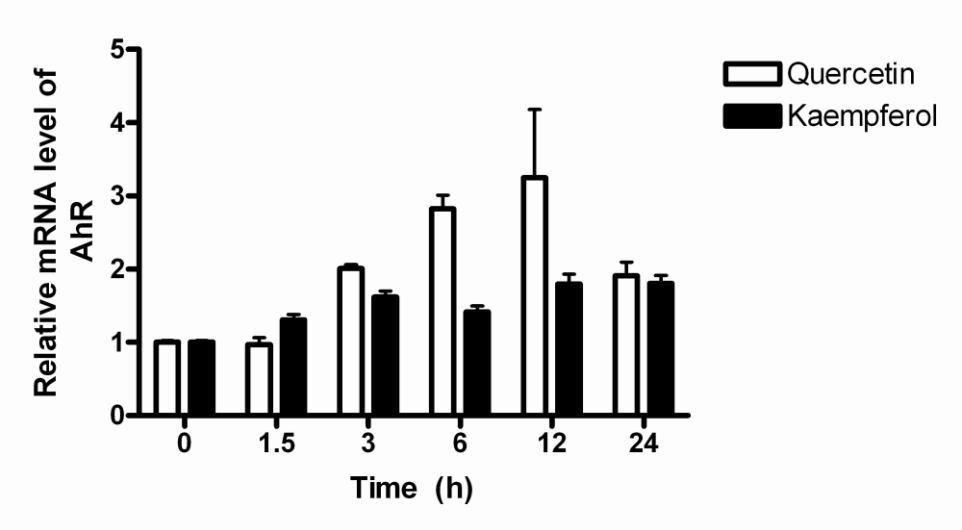


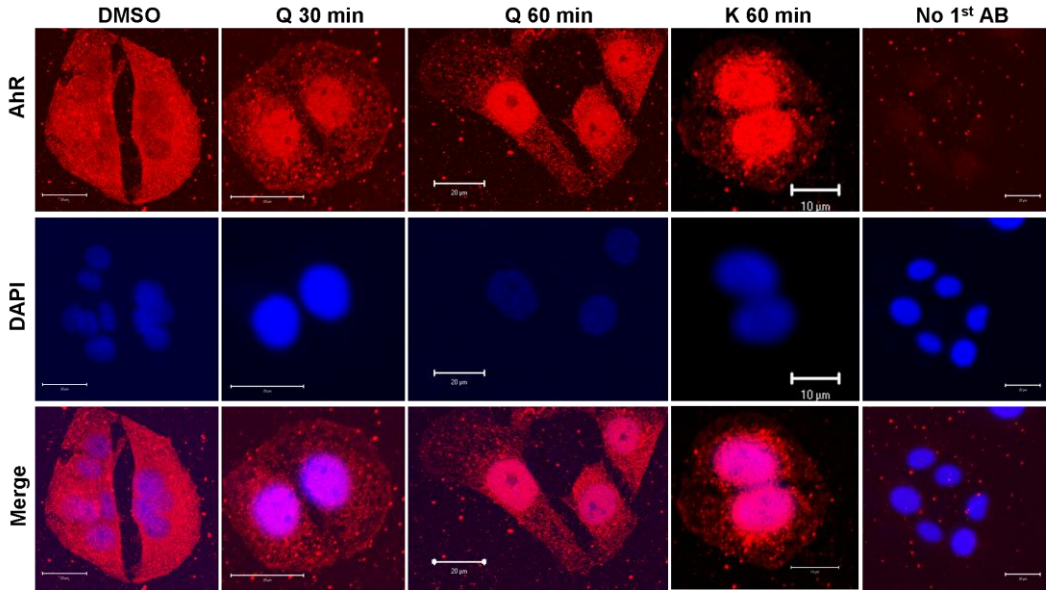
Figure 4.12 Quercetin and kaempferol increase the level of *AhR* mRNA.
Taq-man was performed as described in Figure 3.10 and the mRNA was measured using a primer and probe set for rat *AhR*.

4.2.9 Quercetin and kaempferol act as agonists of AhR

As AhR is a ligand-activated receptor which is controlled by regulating its subcellular localization, immunocytochemistry was carried out to examine whether quercetin and kaempferol could change the localization of AhR in RL-34 cells. These liver epithelial cells were treated for either 30 or 60 min with polyphenols and immunocytochemistry was carried out as described in 2.3.4. The data obtained showed that the portion of AhR protein in cytoplasm of RL-34 cells was relatively higher than that observed in the nucleus. However, upon treatment for 30 min with quercetin, the portion of AhR protein in the nucleus was much higher than that in the

cytoplasm, and when the treatment was extended to 60 min, the portion of AhR protein in the nucleus became even more pronounced. These results suggested that quercetin at 20 μmol/l could stimulate the translocation of AhR from the cytoplasm of RL-34 cell to the nucleus.

A



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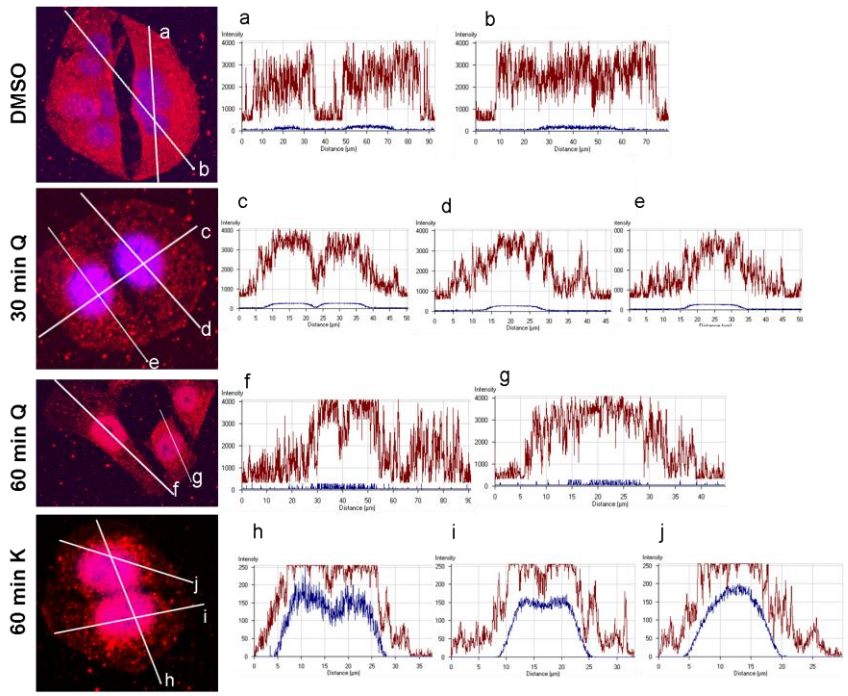


Figure 4.13 Quercetin and kaempferol stimulate translocation of AhR from the cytoplasm to nucleus

Sample preparation, immunocytochemistry and confocal imaging were the same as that described in Figure 4.7 to examine the cellular localization of AhR protein. RFP-labelled secondary antibody was used to locate AhR protein. Nuclear DNA was stained by DAPI. (A) Confocal images of the localization of AhR proteins. (B) Profiling of the distribution of the AhR protein.

4.2.10 Co-IP did not detect physical interaction between Nrf2 and AhR in RL-34 cells upon exposure to quercetin or kaempferol

To investigate whether Nrf2 and AhR physically interact upon exposure to quercetin or kaempferol, immunoprecipitation was carried out. Cells were treated with quercetin or kaempferol for 2 h and immunoprecipitation was carried out as described in 2.4.6. When proteins were pulled-down with anti-Nrf2 antibody, the immunoprecipitated material was probed with AhR antibody. A negative control denoted in the SDS-PAGE gel as "-C" represents material pulled down with rabbit IgG. In the IP sample, the band corresponding to the Nrf2 protein band was identified by comigration with an Nrf2 protein standard and appeared in the sample pulled down by anti-Nrf2 antibody, but was absent in the "-C" lane; this result suggested that the anti-Nrf2 antibody was relatively specific. When samples were blotted with AhR antibody, no band corresponding to the AhR protein appeared in the IP sample. In addition, samples pulled down by anti-Nrf2 antibody did not have any band that was absent from the "-C". Therefore, by immunoprecipitation using the stated Nrf2 and AhR antibodies, we could not detect a physical interaction between Nrf2 and AhR under either basal conditions or upon exposure to quercetin or kaempferol.

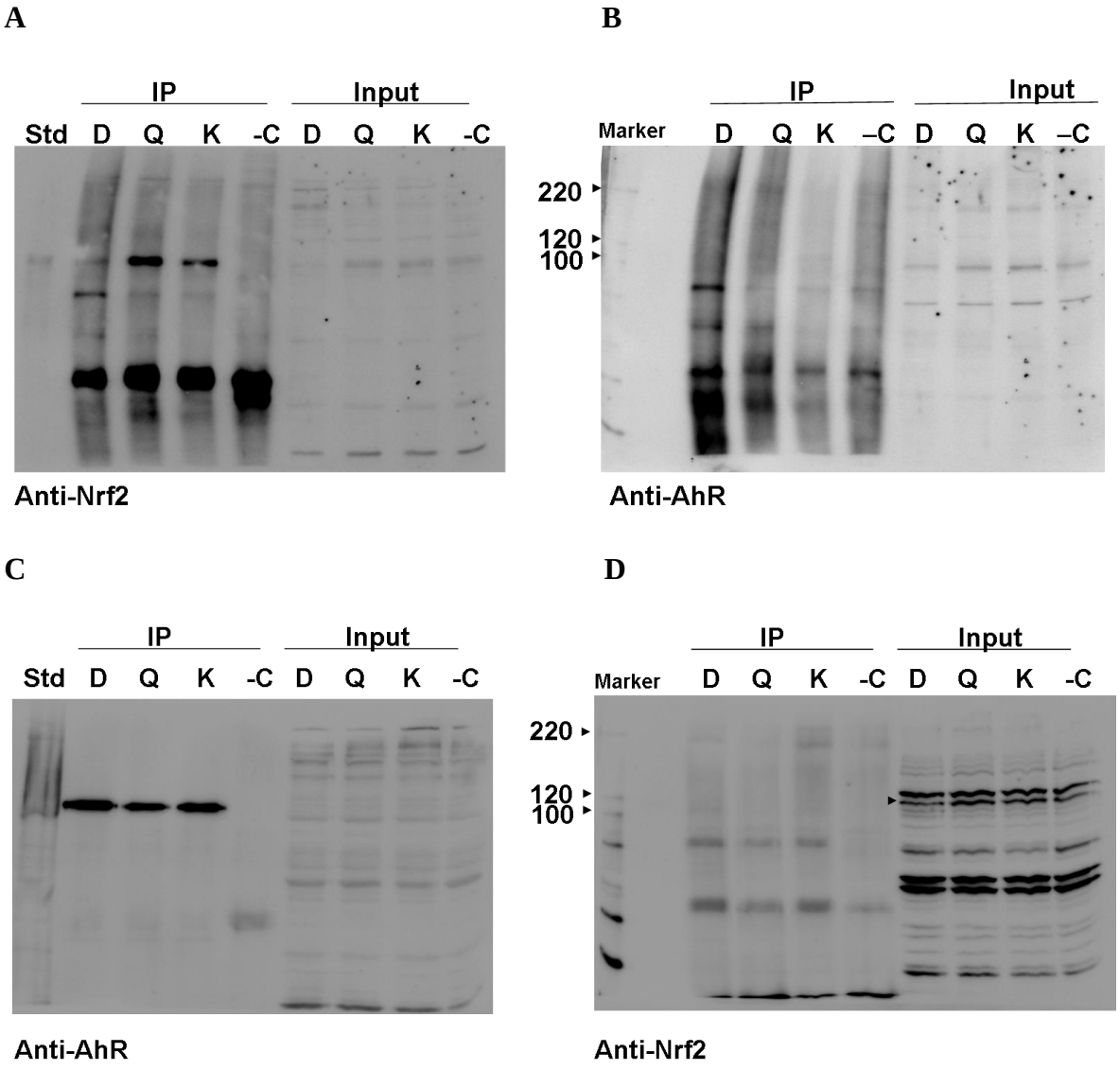


Figure 4.14 Immunoprecipitation indicated there was no physical interaction between Nrf2 and AhR protein either under basal condition or when challenged with quercetin or kaempferol.

RL-34 cells were seeded in duplicate in 100 mm dishes and left to recover and reach to 100% confluence. Cells were then exposed to vehicle DMSO (0.1%, v/v), quercetin (20 μmol/l) or kaempferol (20 μmol/l) for 2 h. Afterwards, cells were subjected for immunoprecipitation as described in material and methods. (A) Proteins were pulled down and probed with Nrf2 antibody. "–C" represented the sample that was pulled down with rabbit IgG. (B) The membrane used for A was stripped and probed with anti-AhR antibody raised in goat (M-20, Santa Cruz). (C) Proteins were pulled down with AhR antibody (M-20, Santa Cruz) and probed with monoclonal anti-AhR antibody (Abcam). "–C" represented the sample that pulled down with goat IgG. (D) The membrane used for (C) was stripped and probed with anti-Nrf2 antibody. Arrow in figure D in the "Input" part indicated the bands corresponding to Nrf2 protein.

4.3 Discussion

4.3.1 Stabilization of Nrf2 protein by quercetin and kaempferol

From the literature, it is known that Nrf2 is subject to rapid degradation under normal homeostatic conditions and its half-life is only around 15 mins in HepG2 cells. Results from our previous experiments suggested that induction of NQO1 by quercetin and kaempferol is mediated through the CNC-bZIP transcription factor Nrf2. Therefore, we hypothesized that quercetin and kaempferol exert a positive effect on Nrf2. To test this hypothesis, we examined Nrf2 protein levels in both RL-34 and MEF cells after exposure to quercetin and kaempferol. This showed that the flavonoids increased the Nrf2 protein levels. Subsequently, we investigated whether this elevation at protein level was due to increased transcription of its gene. It turned out that neither of the flavonoids had any effect on the mRNA level of *Nrf2*. Thus, it appears the elevation in Nrf2 protein is due to increased stabilization of the protein itself. Using the CHX assay, it was found that quercetin and kaempferol increased the half-life of Nrf2 protein in RL-34 cells from ~30 min to 56 and 60 mins, respectively. However, other study showed that the half life of Nrf2 is 15 min in HepG2 (Nguyen *et al.*, 2003) and Hepa cells (Stewart *et al.*, 2003), indicating that quercetin and kaempferol both stabilized the CNC-bZIP protein. Meanwhile, this result also suggested that the half-life of Nrf2 may vary between different cell lines. To further investigate how quercetin and kaempferol stabilized Nrf2, two pathways were examined. Firstly, we checked whether quercetin can inhibit the ubiquitination of Nrf2 which is one of the main mechanisms by which Nrf2 is degraded.

Transfection experiments were carried out in COS-1 cells using cDNAs encoding Nrf2, ubiquitin and Keap1, mimicking the homeostatic condition in cells. The results from these experiments showed that quercetin and kaempferol inhibited the ubiquitination of Nrf2 by ~50%.

Furthermore, it has been proposed from previous experiment involving transient transfection of Keap1 that the BTB-Kelch protein binds and tethers Nrf2 in the cytoplasm where it targets the CNC-bZIP factor for ubiquitination. However other studies have shown that Nrf2 controls the basal expression of its target genes indicating it exhibits a constitutive function and is presumably present in the nucleus under homeostatic conditions. To confirm the cellular localization of Nrf2 protein under homeostatic condition and examine whether quercetin and kaempferol can change such localization, subcelluar fractionation and immunocytochemistry were carried out. Both methods showed that the Nrf2 protein resides predominantly in the nucleus under both homeostatic and stress conditions. In addition, quercetin and kaempferol caused Nrf2 protein to accumulate in the cell.

Taken together, the experiments described in this chapter show that the increase of Nrf2 protein level stimulated by quercetin and kaempferol is through increase in its stability, which results from inhibition of its ubiquitination. Moreover, the flavonoids induced accumulation of Nrf2 proteins principally in the nucleus of cells rather than in the cytoplasm.

4.3.2 Quercetin and kaempferol as agonist of AhR

Overexpression of AhR in RL-34 cells can further increase luciferase activity driven by the mouse Nqo1 gene promoter under both basal and stress conditions, indicating that this transcription factor is constitutively active and can be further activated by quercetin and kaempferol. In addition, our earlier experiment showed that quercetin and kaempferol can increase the mRNA level of *CYP1A1* which is a major target of the AhR, again indicating that the activation of AhR can be induced by these two flavonoids. Although our results showed that mRNA of *AhR* can be increased by quercetin and kaempferol, Western blotting showed there was no change in the protein level of AhR, but such a lack of consistency may be due to the poor sensitivity of the AhR antibody. Activation of AhR can be induced by ligand binding, leading to its translocation from cytosol to nucleus. To examine whether quercetin and kaempferol may act as agonists of the AhR, immunocytochemistry was carried out and this showed that both flavonoids stimulated the nuclear translocation of AhR just after 30 min of exposure to the phytochemicals. As previous studies have shown different effect of quercetin and kaempferol on AhR either as agonists or antagonists indicating their agonist/antagonistic activity is dose- and cell context-dependent. Our result showed these two chemicals both posses agonist activity in RL-34 cells at a concentration of 20 μmol/l.

4.3.3 Interaction between AhR and Nrf2 transcription factor

Cross talk between Nrf2 and AhR has been suggested from previous studies. One of

the possible mechanisms for cross talk between these two transcription factors is through a physical interaction, which has not been reported in previous studies. In our study, compared with over-expression of one transcription factor alone, the over expression of both Nrf2 and AhR did not produce a synergistic effect on the luciferase activity driven by the Nqo1 upstream region after treatment by flavonoids, but rather an additive effect was observed, indicating that neither quercetin nor kaempferol stimulated cross talk between the two factors. Subsequently, we examined whether these two transcription factors interact by immunoprecipitation under normal homeostatic conditions or upon exposure to the flavonoid. The results showed that under either condition, no physical interaction between the AhR and Nrf2 was observed.

4.4 Conclusion

In this chapter, we confirmed the involvement of Nrf2 and AhR protein in the induction of NQO1 by the flavonoids quercetin and kaempferol. Subsequently, we examined the effect that quercetin and kaempferol exerted on transcription factors Nrf2 and AhR. This showed that both flavonoids can increase the stability of Nrf2 and at the same time inhibit its ubiquitination leading to the nuclear accumulation of Nrf2 protein in the cell. The two flavonoids appeared to increase the mRNA level for AhR and could also act as agonists of the receptor at concentration of 20 μmol/l. However, no physical interaction between the two transcription factors was observed neither under homeostatic conditions nor after cells were exposed to the flavonoids

in RL-34 cells.

4.5 Supplementary data: Optimisation of AhR siRNA

The data show that at the final concentration of 40 nM, AhR siRNA knocked down AhR mRNA to 25% while scrambled siRNR did not affect the mRNA level of AhR significantly at this concentration. Thus, 40 nM AhR siRNA was used to knock down *AhR*.

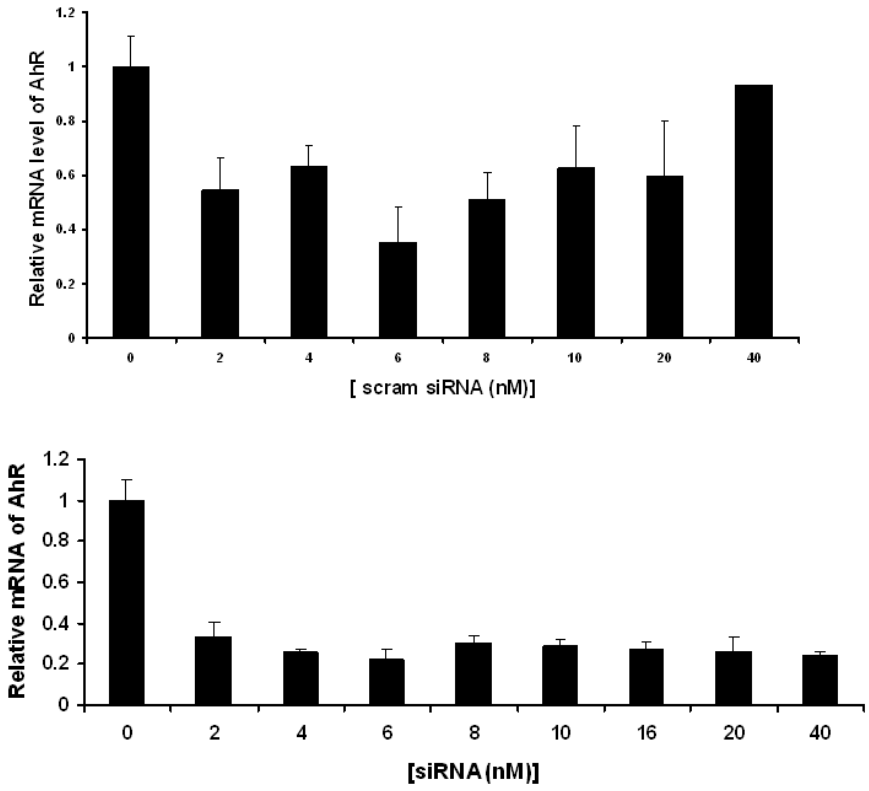


Figure 4.15 Optimization of knocking down AhR using siRNA

RL-34 cells were seeded in 60 mm dish and recovered for ~16 h to reach the confluence of 70%. Cells were then transfected with a range of concentration of siRNA as indicated on the figure. After 16 h transfection, medium was replaced with normal growth medium and cells were recovered for another 24 h before mRNA was extracted. mRNA was measured and analysed as described in figure 3.10. Data represent mean ± standard error.

5 Regulation of antioxidant and detoxification enzymes in mouse by quercetin and kaempferol

5.1 Introduction

Figure 4.16 Safe side of quercetin

Early studies of the safety of flavonoids using Salmonella typhimurium TA98 with S9 mix, demonstrated that quercetin was mutagenic in bacteria (Bjeldanes & Chang, 1977). Subsequently, quercetin was shown to serve as a carcinogen in rat intestine and bladder (Pamukcu *et al.*, 1980). By contrast, a later study using golden hamsters found that treatment with up to 10% quercetin in the diet for ~700 days did not produce an increase in tumours, suggesting it is not carcinogenicity (Morino *et al.*, 1982). In a recent study, Swiss mice were fed quercetin for 28 days at dose of 0, 30, 300, and 3000 mg/kg body weight/ day. No toxicity effect was observed for the treated group compared with control group (Ruiz *et al.*, 2009).

In addition, several studies have examined the safety of quercetin in humans. In 1975, it was reported that a single oral dose of up to 4 g of quercetin had no adverse effects in humans. A phase I clinical trial of quercetin suggested that a bolus of 1400 mg/m^2 (approximately 2.5 grams in a 70 kg adult) weekly dose of quercetin was safe (Ferry *et al.*, 1996). Therefore, it appeared that treatment of quercetin at low concentrations to animals is not toxic.

5.1.1 Bioavailability and metabolism of quercetin

There is more detailed information on quercetin than there is for kaempferol. In this thesis, I will present the information provided by previous studies on the bioavailability and metabolism of quercetin.

Early studies reported that quercetin disappears rapidly from the plasma when administered intravenously to rodents indicating that it does not accumulate in tissues and biological fluids. Besides, it was proposed previously that quercetin is excreted into the faeces without intestinal absorption (Akira *et al.*, 2008). A study by Kuhnau in 1976 and an investigation by Griffths in 1982 suggested quercetin, the aglycone form, but not its glycosides, was taken up in the gastro-intestinal tract by passive diffusion (Griffiths, 1982; Kühnau, 1976). A later study with human volunteers showed that quercetin glycosides can be absorbed in the small intestine, but also that this absorption greatly surpasses that of the aglycone, i.e. 52% of the glycosides was absorbed versus 24% of the aglycone (Hollman *et al.*, 1995). Subsequently, it was confirmed by other studies that the absorption of quercetin is considerably enhanced by its conjugation with a sugar group (Erlund *et al.*, 2000; Hollman *et al.*, 1997). Two possible mechanisms could account for the increased absorption, resulting in a higher plasma peak concentration and in increased bio-availability. Firstly, it is the deglycosylation by β-glucosidases which are capable of liberating free quercetin for passive diffusion (Day *et al.*, 1998; Németh *et al.*, 2003). Secondly, quercetin is obsorbed through the facilitation of carrier-mediated transport. The transporter

responsible for quercetin uptake might be the sodium-dependent glucose transporter-1 (Gee *et al.*, 1998; Walgren *et al.*, 2000) or the multi-drug-resistance protein 2 (Walgren *et al.*, 2000). After absorption, quercetin is metabolized in various organs including the small intestine, colon, liver and kidney. Methylated, sulphated and glucuronidated quercetin are the main metabolites in the small intestine and liver that arise as a result of the actions of drug-metabolizing enzymes (Hollman & Katan, 1997). In addition, a study in a cell model using Caco-2 cells demonstrated that quercetin can be absorbed from the digestive tract and undergo subsequent metabolic conversion (Murota *et al.*, 2000).

Regarding the plasma concentration of quercetin, it is usually in the low nanomolar range, but upon supplementation it may increase to high nanomolar or low micromolar range (Hollman *et al.*, 1996). A study examining its distribution in tissues in rat and pigs showed that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites was found in rat lungs and pig liver and kidney (de Boer *et al.*, 2005). Compared with quercetin aglycone, the plasma concentrations of quercetin metabolites are rather high, ranging from 0-4 μmol/l, indicating that, upon repeated quercetin supplementation, they could attain a considerable plasma level (Hollman *et al.*, 1997; Manach *et al.*, 2005).

5.1.2 NQO1 in animal

Nqo1, regarded as a prototypical Nrf2-target gene, is a cytosolic flavoprotein

catalyzing the two-electron reductive metabolism and detoxification of endogenous and exogenous chemicals. NQO1 and GST were greatly elevated in cytosol from liver and extrahepatic tissues of rodents that were fed the anticarcinogenic dietary antioxidants butylated hydroxyanisole and ethoxyquin. Such cytosols also eliminated the mutagenic activities of urine of mice treated with benzo[α]pyrene. These findings led to the explicit suggestion that induction of drug-metabolizing enzymes could play a major role in protection against neoplasia and toxicity (Itoh *et al.*, 1997; Ramos-Gomez *et al.*, 2001). Double knockout mice deficient for Nqo1 and Nqo2 showed a significantly higher skin tumour frequency and multiplicity compared with control wild-type or single knockout mice (Shen *et al.*, 2010). Nqo1 is widely distributed in various tissues in human. By IHC, Nqo1 has been detected in the respiratory epithelial cells, epithelium of other tissues including breast duct, thyroid follicle, colon, and in the eye in corneal and lens epithelia. In addition, Nqo1 is highly expressed in hepatic tissue and gastrointestinal tract (Siegel & Ross, 2000).

5.1.3 Aims

Earlier studies during this project showed that quercetin and kaempferol could induce ARE-driven and XRE-driven genes expression in RL-34 cells. Therefore, it was important to know whether these flavonoids could act the same way in animals. Thus quercetin or kaempferol were administered to mice for four consecutive days and liver, stomach and intestine were harvested, and the changes in gene expression analysed.

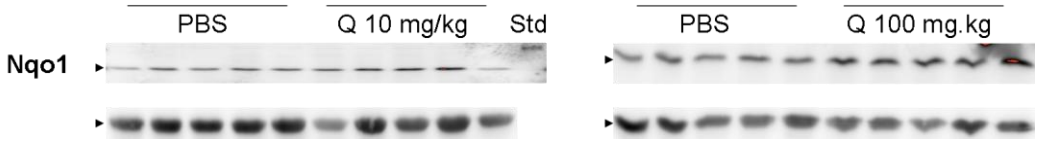
5.2 Results

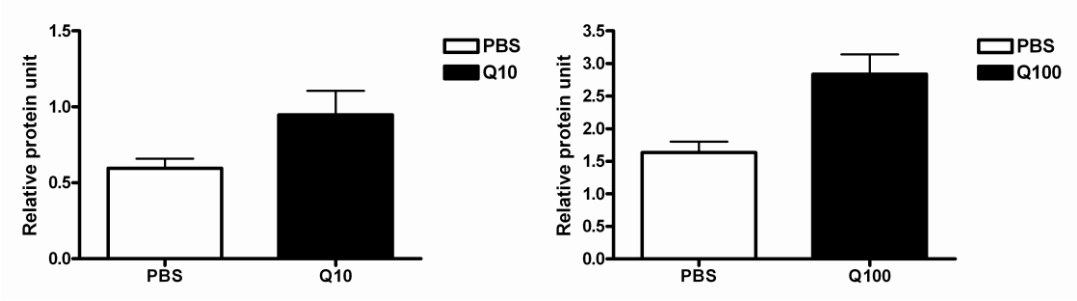
5.2.1 Effects of quercetin and kaempferol on Nqo1 expression in the small intestine

5.2.1.1 Western blotting

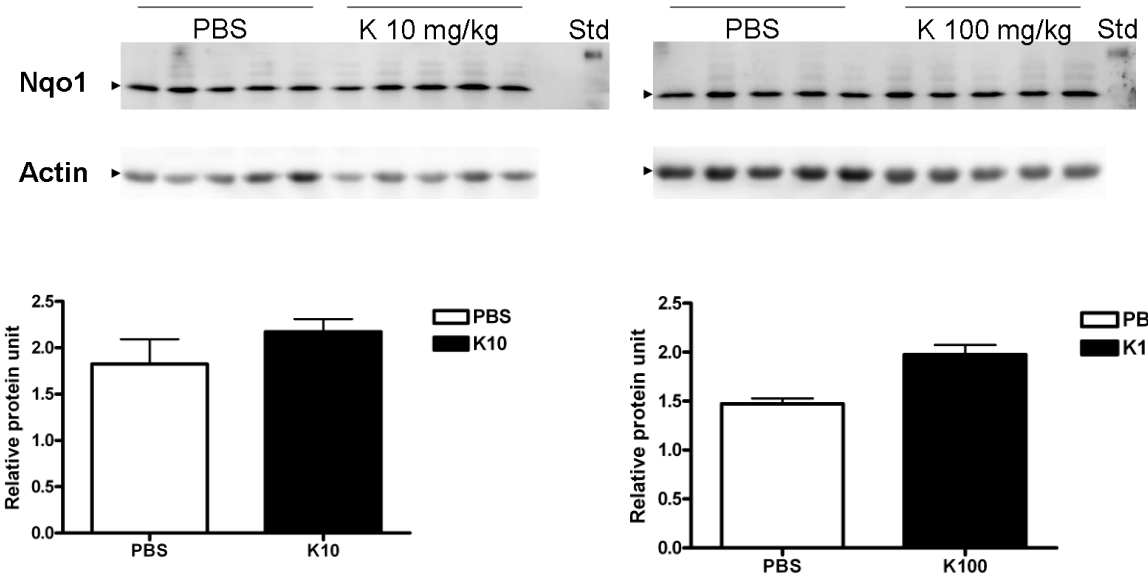
Protein from the small intestine of C57BL/6 mice was extracted as described in 2.4.3.2 and 2.2.6.2 and the level of Nqo1 protein and Nrf2 proteins measured by Western blotting. Densitometry analysis showed that Nqo1 protein level was increased 1.5-fold and 1.8-fold, respectively, by quercetin at a concentration of 10 mg/kg or 100 mg/kg induced Nqo1 (Figure 5.1 A). In the case of kaempferol, the protein level of Nqo1 was increased to 1.2-fold and 1.3-fold, respectively, at the concentration of 10 and 100 mg/kg (Figure 5.1 B). Regarding the protein level of Nrf2, 4-, and 1.5-fold was observed, respectively for the treatment with quercetin at concentration of 10 and 100 mg/kg (Figure 5.1 C). Kaempferol gave an increase of the Nrf2 protein level of 2.2- and 1.8-fold, respectively at the concentration of 10 and 100 mg/kg which is not consistent with the result observed for the change of Nqo1 protein expression by the flavonoids (Figure 5.1 D).

A

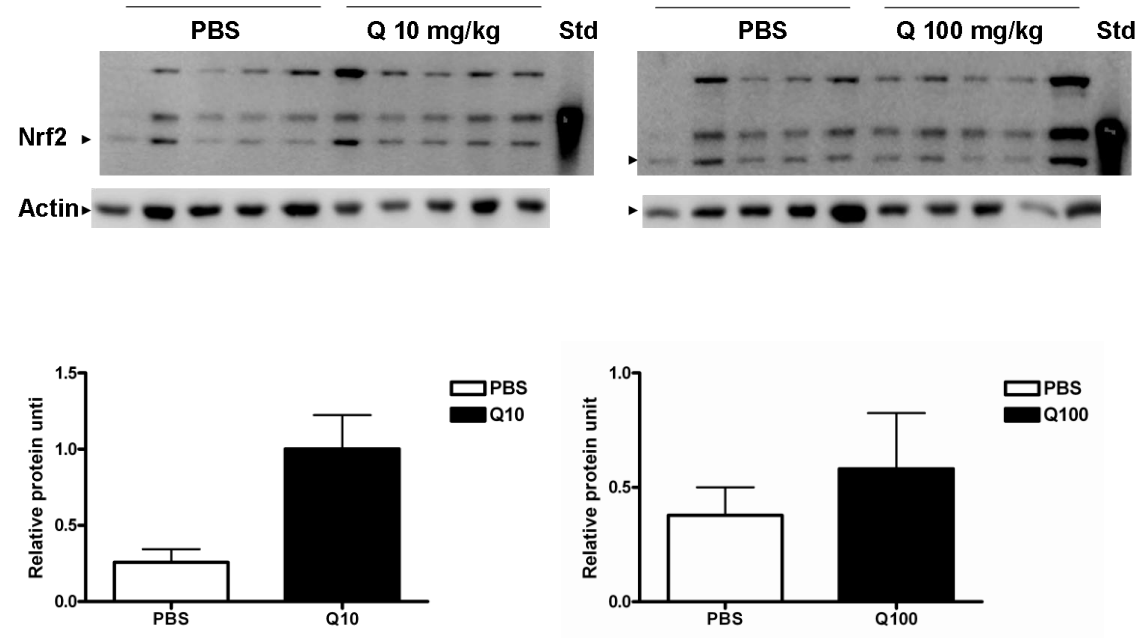



Relative protein unit
1.5
1.0
0.5
0.0
PBS
Q10
PBS
Q10
Relative protein unit
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Q100
PBS
Q100

B


PBS
K 10 mg/kg
Std
PBS
K 100 mg/kg
Std
Nqo1
Actin
Relative protein unit
2.5
2.0
1.5
1.0
0.5
0.0
PBS
K10
PBS
K10
Relative protein unit
2.5
2.0
1.5
1.0
0.5
0.0
PBS
K100
PBS
K100

C


PBS
Q 10 mg/kg
Std
PBS
Q 100 mg/kg
Std
Nrf2
Actin
Relative protein unti
1.5
1.0
0.5
0.0
PBS
Q10
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Q10
Relative protein unit
1.0
0.5
0.0
PBS
Q100
PBS
Q100

D

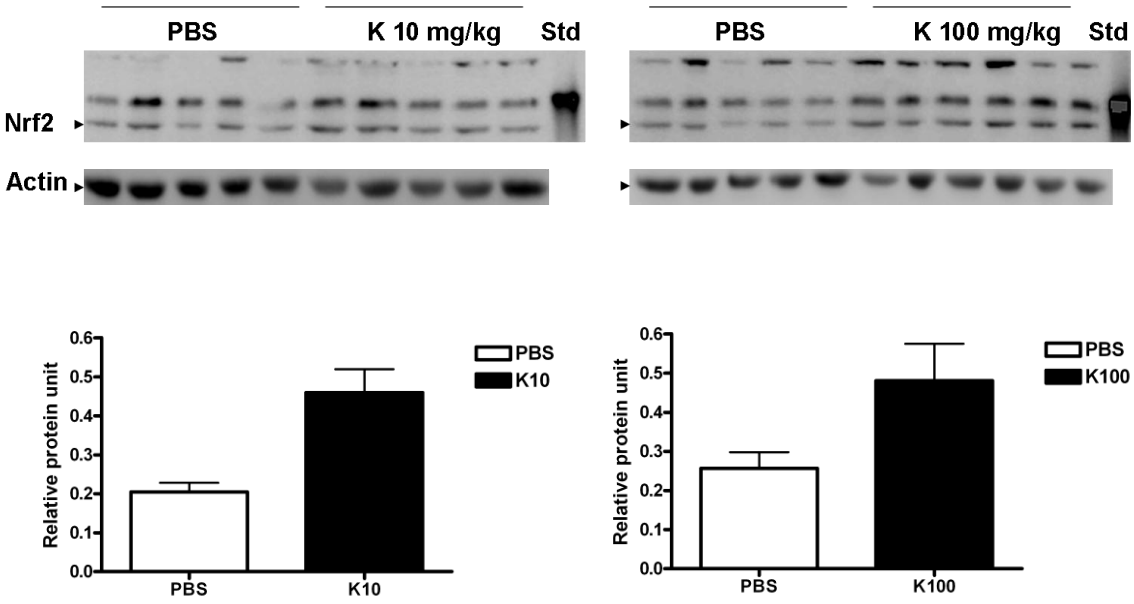


Figure 5.1 Level of Nqo1 and Nrf2 protein in the intestine of mouse.

Mice were treated with 10 or 100 mg/kg quercetin (A&C) or kaempferol (B&D) as described in Materials and Methods. Protein from small intestine was extracted and subjected for the analysis of protein level of Nqo1 (A & B) and Nrf2 (C&D). Membranes were stripped and probed with Actin as well to ensure the equal loading of each sample. Densitometry analyses were carried out. The density of the band corresponding to target protein was normalized to actin and present as the "relative protein unit". Data were then graphed using Graphpad Prism and represent the mean ± standard error.

5.2.1.2 Flavonoids have no effect on the level of *Nqo1* mRNA

To further investigate whether quercetin can exert an effect on *Nqo1* mRNA, Taq-Man was carried out. mRNA from small intestine was extracted as described in 2.2.6.2 and measured by Taq-Man against *Nqo1*. This showed that quercetin with the concentration of 10 mg/kg or 100 mg/kg did not give any significant induction of *Nqo1*. As quercetin behaved as a more potent inducer in cell culture experiment and it showed no effect on the mRNA level of *Nqo1* in the small intestine of mice, the

same experiment was not carried out for kaempferol.

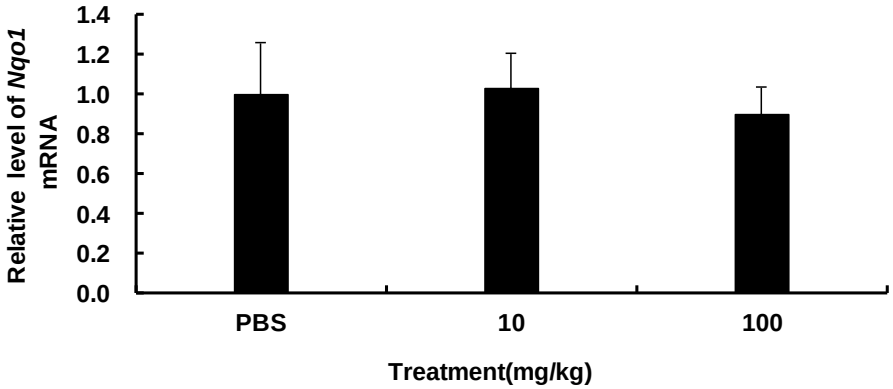


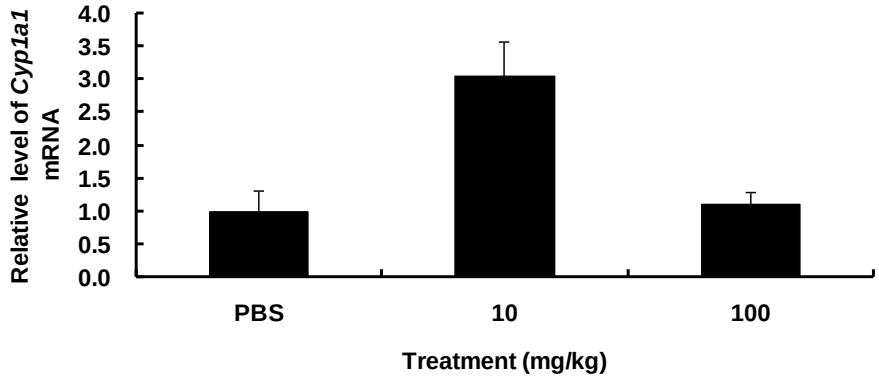
Figure 5.2 Relative mRNA level of NQO1 in the small intestine of mouse

Mice were treated with 10 or 100 mg/kg quercetin and mRNA was extracted from the small intestine as described in Materials and Methods. Triplicate reactions were carried out for each sample and normalized to the value of actin. Data presented here are the mean value of the 5 sample of each group. Mean value of each group was compared to that of the PBS group which was set as one. Mean ± Standard deviation was also presented for each group.

5.2.2 Quercetin can increase the mRNA level of *Cyp1a1* in small intestine but has no effect on *AhR*

mRNA samples from small intestine extracted earlier were also used to measure the mRNA level of *Cyp1a1* by Taq-Man. This showed that quercetin at 10 mg/kg increased the mRNA level of *Cyp1a1* about ~ 3-fold. However, when the mice were treated with 100 mg/kg quercetin, there was no change in mRNA level of *Cyp1a1* was observed (Figure 5.3 A). The level of *AhR* mRNA was also examined and it showed that the flavonoids have no effect (Figure 5.3 B).

A



B

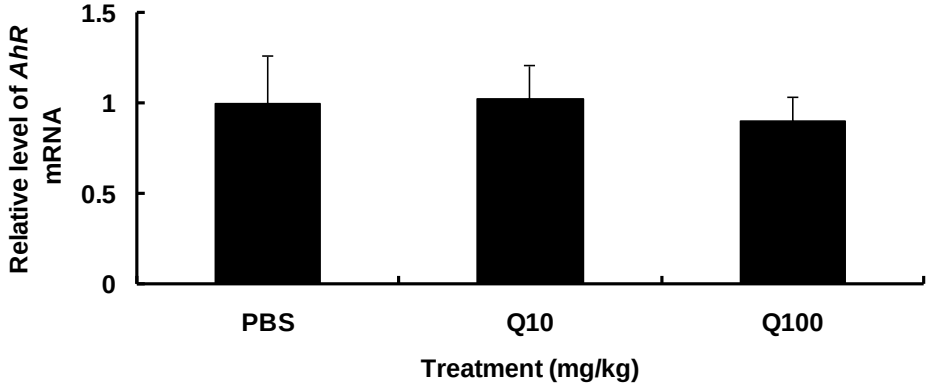


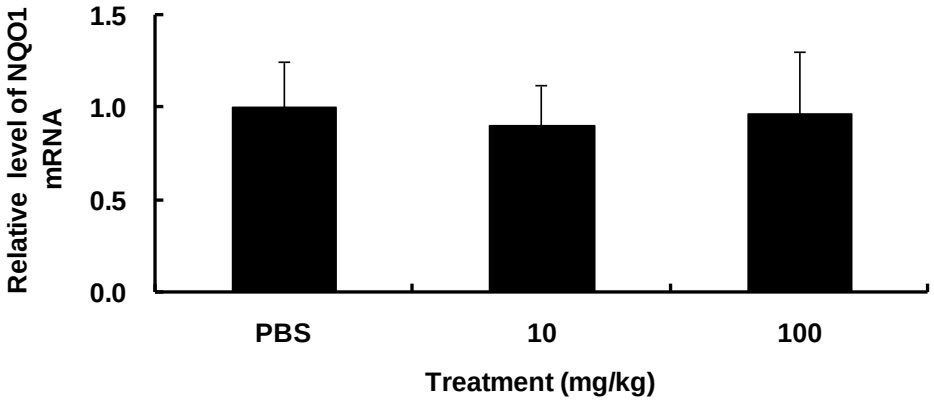
Figure 5.3 Relative levels of *Cyp1a1* and *AhR* mRNA in the small intestine of mouse

Mice were treated with 10 or 100 mg/kg quercetin and mRNA was extracted from the small intestine as described in material and methods. Triplicate reactions were carried out for each sample and normalized to the value of actin. Data presented here are the mean value of the 5 sample of each group. Mean value of each group was compared to that of the PBS group which was set as one. Mean ± Standard deviation was also presented for each group.

5.2.3 Quercetin had no effect on the mRNA level of *Nqo1* or *Nrf2* in liver

mRNA from liver was extracted as described earlier and was measured by Taq-man against *Nqo1* and *Nrf2*. It showed that quercetin at either 10 or 100 mg/kg had any effect on the mRNA level of *Nqo1* (Figure 5.4 A). In the case of *Nrf2*, and at both concentrations quercetin decreased the mRNA level to ~75% (Figure 5.4 B).

A



B

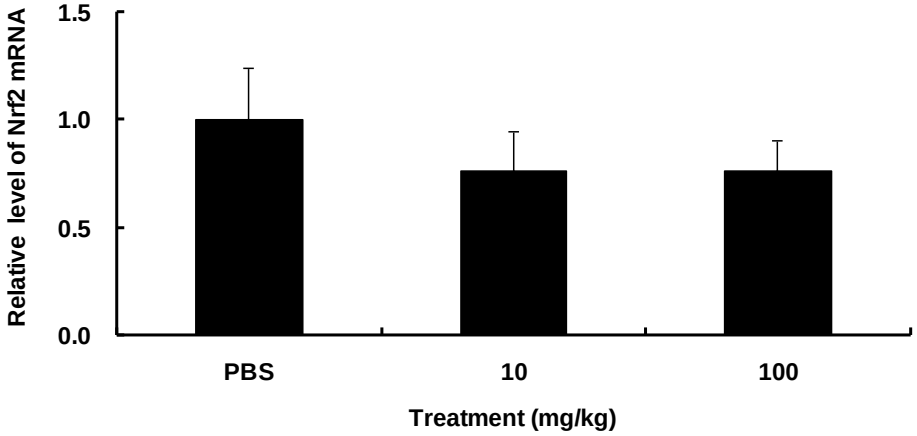


Figure 5.4 Relative mRNA level of *Nqo1* or *Nrf2* in the liver of mouse

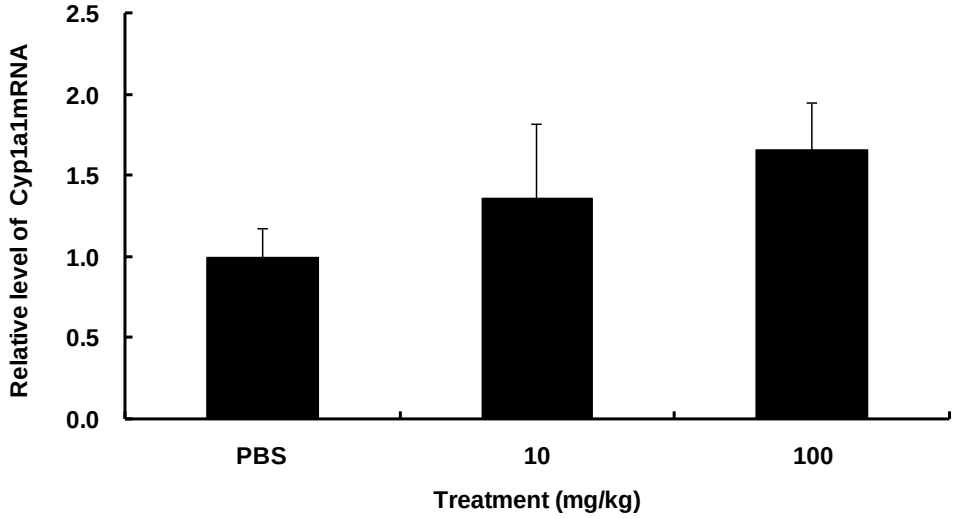
Mice were treated with 10 or 100 mg/kg quercetin and mRNA was extracted from the liver as described in material and methods. Triplicate reactions were carried out for each sample and normalized to the value of actin. Data presented here are the mean value of the 5 sample of each group. Mean value of each group was compared to that of the PBS group which was set as one. Mean ± Standard deviation was also presented for each group.

5.2.4 Quercetin can increase the mRNA level of CYP1A1 and AhR in liver

The mRNA was extracted from livers of mice treated with either PBS, or 10 or 100 mg/kg quercetin. As shown in Figure 5.5, quercetin at 10 mg/kg caused induction of *Cyp1a1* gene of around 1.5-fold (Figure 5.5 A) but had no effect on AhR (Figure 5.5

B). When mice were treated with 100 mg/kg quercetin, there was a ~1.7 fold increase of the CYP1A1 mRNA and 1.5 fold for the mRNA of AhR.

A



B

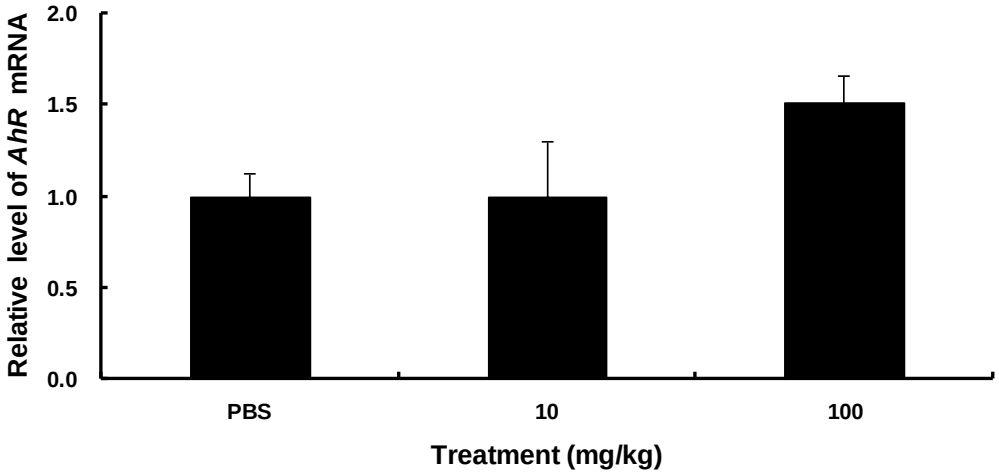


Figure 5.5 Relative mRNA level of CYP1A1 and AhR in the liver of mouse treated with quercetin

Mice were treated with 10 or 100 mg/kg quercetin and mRNA was extracted from the small intestine as described in Materials and Methods. Triplicate reactions were carried out for each sample and normalized to the value of actin. Data presented here are the mean value of the 5 sample of each group. Mean value of each group was compared to that of the PBS group which was set as one. Mean ± Standard deviation was also presented for each group.

5.3 Discussion

5.3.1 Effect of the flavonoids on the expression of Nqo1 and Nrf2 in small intestine and liver

Our previous studies showed that quercetin and kaempferol can increase the Nqo1 and its transcription factor Nrf2 in cell culture experiments. Therefore experiments were carried out using an animal model to examine whether the flavonoids could exert similar effect in vivo. Four days of consecutive treatment of quercetin or kaempferol at non-toxic concentration to C57/BL6 male mice appeared to increase the amount of protein level of Nqo1 by 20-80% in the small intestine. Indeed a dose-response was observed with quercetin at 100 mg/kg giving the highest induction of 80%. Such increase was accompanied with the increase of Nrf2 protein. Unexpectedly, the increase in Nrf2 protein is much higher than that of its target protein Nqo1. In addition, it was found that a low dose of each treatment of 10 mg/kg gave higher induction than the higher dose which was not consistent with the induction of Nqo1 by quercetin and kaempferol. In addition, a large variation between individual mice was observed in levels of Nqo1 and Nrf2 protein. Other reasons for the inconsistencies in these results are that as the increase in the Nqo1 protein is not large enough for the densitometry analysis to give accurate measurements. Subsequently, to confirm whether Nqo1 was induced, *Nqo1* mRNA level was measured and this showed that there was no significant change. Two possibilities may explain such contradiction. Firstly, the tissues were harvested 24 h

after treatment, while our cell culture result showed that quercetin and kaempferol gave the highest induction of *NQO1* mRNA at 12 h. This finding suggests that the induction of Nqo1 or Nrf2 by quercetin or kaempferol may be present in some individuals will need to be confirmed using a larger group of animals and a different time point. Our cell culture study showed that quercetin and kaempferol gave the highest induction of *NQO1* mRNA at 12 h, whilst the mRNA analyzed for animal study was extracted from tissues harvested after 24 h of treatment. Such a difference may account for failure to detect the induction of *Nqo1* mRNA. Secondly, densitometry analysis used for Western blotting analysis is semi-quantitative and therefore may not be sufficiently accurate to represent the levels of protein expression, especially when the change is moderate. To overcome such quantifying problems, enzyme activity could be measured to indicate the chage more accurately.

Taken together, to achieve a better assessment of whether flavonoids affect the expression of drug metabolizing enzymes, a larger group of animals maybe useful. In addition, synthetic diet which does not contain any antioxidant should be considered, as the diet itself may contain polyphenols that mask the effect of quercetin on gene expression. Another strategy to avoid the effect of diet is to try a synthetic one.

5.3.2 Effect of quercetin on the mRNA level of *Cyp1a1*

As all the previous study showed that quercetin exerted more potent effect than kaempferol on the expression of Nqo1 and Nrf2, we only carried on the experiment

to examine the effect of quercetin on *Cyp1a1* and its transcription factor *AhR*. This showed that quercetin can increase the mRNA level of *Cyp1a1* in small intestine at the low dose of 10 mg/kg but not at the higher dose 100mg/kg. In addition, the mRNA level of its transcription factor was not affected by quercetin. In liver, the mRNA levels of both *Cyp1a1* and *AhR* were elevated by quercetin which is consistent with results achieved from the cell culture study. However, compared with the induction of *Cyp1a1* in the small intestine, around 3-fold, the induction for CYP1A1 at the same dose 10 mg/kg is much lower 20%. Such low induction may not be significant to lead to any biological consequence. However, these need to be further studied.

Previous literature have shown that activation of AhR and its target gene Cyp1a1 leads to the biotransformation of polycyclic aromatic hydrocarbons into active genotoxic metabolites, resulting in the initiation of chemical carcinogenesis (Nebert & Dalton, 2006). However, cell culture experiments have shown that quercetin can compete with TCDD for binding to the AhR thereby decreasing Cyp1a1 induction by TCDD, and in turn resulting in decreased toxicity.

In addition to its ability to act as a transcription factor, AhR also functions as a ligand-dependent E3 ubiquitin ligase of certain nuclear receptors. Kawajiri *et al.* (2010) have reported that activation of AhR by natural ligands such as indole derivatives suppresses intestinal tumour development in the $APC^{min/+}$ mouse

(Fujii-Kuriyama & Kawajiri, 2010). Based on our observation that quercetin can stimulate an increase in *Cyp1a1* mRNA, it is possible that flavonoids can prevent intestinal carcinogenesis by activating AhR.

6 Discussion and future perspectives

6.1 Flavonols' effect on the Keap1/Nrf2/ ARE gene battery

Previous studies on flavonoids focus on their effect on the progress of various diseases such as cancer, cardiovascular disease and neurodegenerative diseases. Our work firstly examined whether and how they could provide protective effect to normal cells, RL-34 and MEF cells, by studying their effect on the Keap1/Nrf2/ARE gene battery, which are involved in antioxidant and detoxification system of the human body. Quercetin and kaempferol, the flavonols, were found the most potent ARE-inducers compared with the other flavonoids tested in the study. Further data in the Chapter 3 and 4 of this thesis provided evidence of the effect that quercetin and kaempferol exerted on the Keap1/Nrf2/ARE gene battery. The results showed that these two flavonols can regulate Nrf2 at its protein level by increasing its stability and inducing cellular translocation from cytosol to nucleus, but not on its mRNA level. Such changes of Nrf2 protein ultimately lead to increased transcription, translation activity of one of its target gene, Nqo1. In addition, our result showed the increased level of Nrf2 protein is due to the inhibition of ubiquitination of Nrf2 by quercetin and kaempferol. How such inhibition was achieved is still unclear. One of the possibilities is that quercetin and kaempferol or their metabolites can modify Keap1, the inhibitor of Nrf2, which in turn weakens the activity of Keap1, leading to the increased stability of Nrf2.

Furthermore, mutagenesis experiments revealed that the ARE in the promoter region of *Nqo1* was required for the basal level and induction of Nqo1 by quercetin and kaempferol.

Although in RL-34 cells quercetin and kaempferol caused good inductions of Nqo1, it had no effect on the expression of Gstp1, a protein encoded by another target gene of Nrf2 (Ikeda *et al.*, 2002; Suzuki *et al.*, 2005). A recent study found that Nqo1 is more inducible than GSTP1 in either normal or immortalized human lung cells, and neither of them were induced in adenocarcinoma A549 cells which have high level of constitutive expression of Nqo1 and GSTP1 (Tan *et al.*, 2010). Another study showed that GSTP1 can be induced by sulforphane in rat clone 9 cells (Lii *et al.*, 2010). Therefore, it is possible that quercetin and kaempferol induce specific cytoprotective genes in a cell specific manner.

6.2 Quercetin and kaempferol's effect on the AhR/XRE gene battery

Previous studies showed that quercetin and kaempferol could act as either agonist or antagonist of AhR depending on concentration and cell context. In our study, we examined quercetin and kaempferol at 20 μmol/l in RL-34 cells and proved that they can act as AhR agonist by immunofloresence. This finding is further supported by the results showing that they can increase the mRNA level of *Cyp1a1*, a target gene of AhR. Such regulation of Cyp1a1 was also observed in the small intestine of male

mice which has been fed with quercetin and kaempferol (10 mg/kg body weight) for four days. Previous study by Kawarjiri, et al (2009) suggested the natural AhR ligand may be used to prevent intestinal cancer (Kawajiri *et al.*, 2009). Therefore, the quercetin and kaempferol may be able to exert similar effect. However, this needs further investigations.

In addition, our study also showed that induction of Nqo1 by quercetin and kaempferol is partly through XRE. By using WT MEF and DBA2/O MEF, we showed that the lack of functional AhR did not alter the transactivation of Nqo1 by quercetin or kaempferol either in the presence or absence of XRE. However, by overexpressing AhR in RL-34 cells, we do find that overexpression of AhR can increase the transactivation of Nqo1 by quercetin and kaempferol. Therefore, it is hard to conclude whether AhR is not involved in the regulation of Nqo1. Further experiments need to be carried out to investigate this question, such as using Hepa1c1c7 and AhR deficient Hepa1c1c7 cells or knocking down AhR in RL-34 or WT MEF cells.

6.3 Co-ordinate regulation of Nqo1 by Nrf2 and AhR

Previous studies have suggested several genes encoding drug metabolizing enzymes can be induced by TCDD, a typical AhR activator in an Nrf2 dependent manner such as UGT1A6, UGT1A10, Nqo1 and Gsta1 (Yeager *et al.*, 2009). The underlying mechanism is probably due to the presence of both ARE and XRE in the promoter

region in these genes (Nioi & Hayes, 2004). The study of regulation of UGTs have identified the presence of XRE and ARE in the promoter region of UGT1A10 and revealed it can be regulated by both Nrf2 and AhR. They also showed that the coordinated regulation of UGT1A10 is not the result of cross reactivity but their simultaneously binding to both the XRE and ARE in the promoter region in UGT*1A10* (Kalthoff *et al.*, 2010). I also studied whether Nrf2 and AhR can coordinately regulate Nqo1. Over expression of either Nrf2 or AhR can up-regulate the transactivation of *Nqo1*, while the over expression of both transcription factors further increased both the basal level and induction of Nqo1 by quercetin and kaempferol, additively but not synergistically. Furthermore, by immunoprecipitation we did not observe any interactions between these two transcription factors. Taken together, ARE but not XRE plays predominated role in the regulation of *Nqo1* by quercetin and kaempferol. Whether Nqo1 is regulated by Nrf2 and AhR in the way UGT1A10 is regulated needs further investigation.

6.4 Chemoprevention and up-regulation of Nrf2 and AhR

In this thesis, evidences have been provided that quercetin and kaempferol could increase the activity of Nrf2 and AhR protein. As we discussed in the introduction chapter, constitutive inactivation of Nrf2 can increase the sensitivity to toxicant and carcinogens while constitutive activation of Nrf2 are observed in the developing adenocarcinoma cells. In addition, increased activation of Nrf2 also contributes to chemoresistance of cancerous cells. Such contradictory roles of Nrf2 are probably

due to its ability to protect both normal cells and cancerous cells. The activation of Nrf2 by quercetin and kaempferol in normal cells can increase the capacity of cells defensive system and therefore can prevent cells from the attack by toxicants. As AhR is involved in many cellular physiological activities, whether the activation of AhR is good or bad still needs more studies.

7 References

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